



Europäisches Patentamt
European Patent Office
Office européen des brevets



Publication number: **0 684 313 A2**

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: **95105693.6**

(51) Int. Cl.⁶: **C12N 15/55, C12N 9/16,
A23K 1/165**

(22) Date of filing: **15.04.95**

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(30) Priority: **25.04.94 EP 94810228**

(43) Date of publication of application:
29.11.95 Bulletin 95/48

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB IT LI NL

(71) Applicant: **F. Hoffmann-La Roche AG**
CH-4002 Basel (CH)

(72) Inventor: **Van Loon, Adolphus**
17 Waldshuterstrasse
CH-4310 Rheinfelden (CH)
Inventor: **Mitchell, David**
2a Kundmannweg
CH-4147 Aesch (CH)

(74) Representative: **Braun, Axel et al**
F.Hoffmann-La Roche AG
Grenzacherstrasse 124
Postfach 32 55
CH-4002 Basel (CH)

(54) **Polypeptides with phytase activity.**

(57) The present invention is directed to a DNA sequence coding for a polypeptide having phytase activity which DNA sequence is derived from specific groups of fungi, polypeptides encoded by such DNA sequences, vectors comprising such DNA sequences, bacteria or a fungal or yeast host transformed by such DNA sequences or vectors, a process for the preparation of a polypeptide by culturing such transformed hosts and composite feeds comprising one or more such polypeptides.

EP 0 684 313 A2

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8) are enzymes that hydrolyze phytate (*myo*-inositol hexakisphosphate) to *myo*-inositol and inorganic phosphate and are known to be valuable feed additives.

A phytase was first described in rice bran in 1907 [Suzuki et al., Bull. Coll. Agr. Tokio Imp. Univ. 7, 495 (1907)] and phytases from *Aspergillus* species in 1911 [Dox and Golden, J. Biol. Chem. 10, 183-186 (1911)]. Phytases have also been found in wheat bran, plant seeds, animal intestines and in microorganisms [Howsen and Davis, Enzyme Microb. Technol. 5, 377-382 (1983), Lambrechts et al., Biotech. Lett. 14, 61-66 (1992), Shieh and Ware, Appl. Microbiol. 16, 1348-1351 (1968)].

The cloning and expression of the phytase from *Aspergillus niger* (ficcum) has been described by VanHartingsveldt et al., in Gene, 127, 87-94 (1993) and in European Patent Application, Publication No. 420 358 and from *Aspergillus niger* var *awamori* by Piddington et al. in Gene 133, 55-62 (1993).

Since phytases used so far in agriculture have certain disadvantages it is an object of the present invention to provide new phytases or more generally speaking polypeptides with phytase activity against inositol phosphates including phytases ("phytase activity") in large quantities with improved properties. Since it is known that phytases used so far loose activity during the feed pelleting process due to heat treatment, improved heat tolerance would be such a property.

So far phytases have not been reported in thermotolerant fungus with the exception of *Aspergillus fumigatus* [Dox and Golden et al., J. Biol. Chem. 10, 183-186 (1911)] and *Rhizopus oryzae* [Howson and Davies, Enzyme Microb. Technol. 5, 377-382 (1993)]. Thermotolerant phytases have been described originating from *Aspergillus terreus* Strain 9A-1 [Temperature optimum 70 °C; Yamada et al., Agr. Biol. Chem. 32, 1275-1282 (1968)] and *Schwanniomyces castellii* [Temperature optimum 77 °C; Segueilha et al., Bioeng. 74, 7-11 (1992)]. However for commercial use in agriculture such phytases must be available in large quantities. Accordingly it is an object of the present invention to provide DNA sequences coding for heat tolerant phytases. Improved heat tolerance of phytases encoded by such DNA sequences can be determined by assays known in the art, e.g. by the processes used for feed pelleting or assays determining the heat dependence of the enzymatic activity itself as described, e.g. by Yamada et al. (s.a.).

It is furthermore an object of the present invention to screen fungi which show a certain degree of thermotolerance for phytase production. Such screening can be made as described, e.g. in Example 1. In this way heat tolerant fungal strains, listed in Example 1, have been identified for the first time to produce a phytase.

Heat tolerant fungal strains, see e.g. those listed in Example 1, can than be grown as known in the art, e.g. as indicated by their supplier, e.g. the American Tissue Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Agricultural Research Service Culture Collection (NRRL) and the Centraalbureau voor Schimmelcultures (CBS) from which such strains are available or as indicated, e.g. in Example 2.

Further improved properties are, e.g. an improved substrate specificity regarding phytic acid [*myo*-inositol (1,2,3,4,5,6) hexakisphosphate] which is a major storage form of phosphorous in plants and seeds. For the complete release of the six phosphate groups from phytic acid an enzyme is required with sufficient activity against phytic acid and all other inositol phosphate molecules. Using e.g. *Aspergillus niger* phytase requires for this complete release the addition of the pH 2.5 acid phosphatase. Having only one enzyme with the required activity would be of clear advantage. For example, International Patent Application Publication No. 94/03072 discloses an expression system which allows the expression of a mixture of phytate degrading enzymes in desired ratios. However, it would be even more desirable to have both such activities in a single polypeptide. Therefore it is also an object of the present invention to provide DNA sequences coding for such polypeptides. Phytase and phosphatase activities can be determined by assays known in the state of the art or described, e.g. in Example 9.

Another improved property is, e.g. a so called improved pH-profile. This means, e.g. two phytin degrading activity maxima, e.g. one at around pH 2.5 which could be the pH in the stomach of certain animals and another at around pH 5.5 which could be the pH after the stomach in certain animals. Such pH profile can be determined by assays known in the state of the art or described, e.g. in Example 9. Accordingly it is also an object of the present invention to provide DNA sequences coding for such improved polypeptides.

In general it is an object of the present invention to provide a DNA sequence coding for a polypeptide having phytase activity and which DNA sequence is derived from a fungus selected from the group consisting of *Acrophialophora levis*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus sojae*, *Calcarisporiella thermophila*, *Chaetomium rectopilum*, *Corynascus thermophilus*, *Humicola* sp., *Mycelia sterilia*, *Myrococcum thermophilum*, *Myceliophthora thermophila*, *Rhizomucor miehei*, *Sporotrichum cellulophilum*, *Sporotrichum thermophile*, *Scytalidium indonesicum* and *Talaromyces thermophilus* or a DNA

sequence coding for a fragment of such a polypeptide which fragment still has phytase activity, or more specifically such a DNA sequence wherein the fungus is selected from the group consisting of *Acrophialophora levis*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus terreus*, *Calcarisporiella thermophila*, *Chaetomium rectopilum*, *Corynascus thermophilus*, *Sporotrichum cellulophilum*, *Sporotrichum thermophile*, *Mycelia sterilia*, *Myceliophthora thermophila* and *Talaromyces thermophilus*, or more specifically such a DNA sequence wherein the fungus is selected from the group consisting of *Aspergillus terreus*, *Myceliophthora thermophila*, *Aspergillus fumigatus*, *Aspergillus nidulans* and *Talaromyces thermophilus*. DNA sequences coding for a fragment of a polypeptide of the present invention can, e.g. be between 1350 and 900, preferably between 900 and 450 and most preferably between 450 and 150 nucleotides long and can be prepared on the basis of the DNA sequence of the complete polypeptide by recombinant methods or by chemical synthesis with which a man skilled in the art is familiar with.

Furthermore it is an object of the present invention to provide a DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence is selected from the following:

- (a) the DNA sequence of Figure 1 [SEQ ID NO:1] or its complementary strand;
- (b) a DNA sequence which hybridizes under standard conditions with sequences defined under (a) or preferably with the coding region of such sequences or more preferably with a region between positions 491 to 1856 of such DNA sequences or even more preferably with a genomic probe obtained by preferably random priming using DNA of *Aspergillus terreus* 9A1 as described in Example 12.
- (c) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with sequences of (a) or (b), but which codes for polypeptides having exactly the same amino acid sequences as the polypeptides encoded by these DNA sequences; and
- (d) a DNA sequence which is a fragment of the DNA sequences specified in (a), (b) or (c).

"Standard conditions" for hybridization mean in this context the conditions which are generally used by a man skilled in the art to detect specific hybridization signals and which are described, e.g. by Sambrook et al., "Molecular Cloning" second edition, Cold Spring Harbor Laboratory Press 1989, New York, or preferably so called stringent hybridization and non-stringent washing conditions or more preferably so called stringent hybridization and stringent washing conditions a man skilled in the art is familiar with and which are described, e.g. in Sambrook et al. (s.a.) or even more preferred the stringent hybridization and non-stringent or stringent washing conditions as given in Example 12. "Fragment of the DNA sequences" means in this context a fragment which codes for a polypeptide still having phytase activity as specified above.

It is also an object of the present invention to provide a DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence is selected from the following:

- (a) the DNA sequence of Figure 2 [SEQ ID NO:3] or its complementary strand;
- (b) a DNA sequence which hybridizes under standard conditions with sequences defined under (a) or preferably a region which extends to about at least 80 % of the coding region optionally comprising about between 100 to 150 nucleotides of the 5' end of the non-coding region of such DNA sequences or more preferably with a region between positions 2068 to 3478 of such DNA sequences or even more preferably with a genomic probe obtained by preferably random priming using DNA of *Myceliophthora thermophila* as described in Example 12.
- (c) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with sequences of (a) or (b), but which codes for polypeptides having exactly the same amino acid sequences as the polypeptides encoded by these DNA sequences; and
- (d) a DNA sequence which is a fragment of the DNA sequences specified in (a), (b) or (c).

"Fragments" and "standard conditions" have the meaning as given above.

It is also an object of the present invention to provide a DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence is selected from the following:

- (a) a DNA sequence comprising one of the DNA sequences of Figures 4 [SEQ ID NO:5], 5 [SEQ ID NO:7], 6 [SEQ ID NO:9] or 10 ["aterr21", SEQ ID NO:13: "aterr58": SEQ ID NO:14] or its complementary strand;
- (b) a DNA sequence which hybridizes under standard conditions with sequences defined under (a) or preferably with such sequences comprising the DNA sequence of Figure 4 [SEQ ID NO:5] isolatable from *Talaromyces thermophilus*, or of Figure 5 [SEQ ID NO:7] isolatable from *Aspergillus fumigatus*, or of Figure 6 [SEQ ID NO:9] isolatable from *Aspergillus nidulans* or of one or both of the sequences given in Figure 10 ["aterr21", SEQ ID NO:13: "aterr58": SEQ ID NO:14] isolatable from *Aspergillus terreus* (CBS 220.95) or more preferably with a region of such DNA sequences spanning at least 80 % of the coding region or most preferably with a genomic probe obtained by random priming using DNA of *Talaromyces thermophilus* or *Aspergillus fumigatus* or *Aspergillus nidulans* or *Aspergillus terreus* (CBS

220.95) as described in Example 12.

(c) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with sequences of (a) or (b) but which codes for polypeptides having exactly the same amino acid sequences as the polypeptides encoded by these DNA sequences; and

5 (d) a DNA sequence which is a fragment of the DNA sequences specified in (a), (b) or (c).

It is furthermore an object of the present invention to provide a DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence is selected from a DNA sequence comprising the DNA sequence of Figure 4 [SEQ ID NO:5] isolatable from *Talaromyces thermophilus*, of Figure 5 [SEQ ID NO:7] isolatable from *Aspergillus fumigatus*, of Figure 6 [SEQ ID NO:9] isolatable from *Aspergillus*
10 *nidulans* or of Figure 10 ["aterr21": SEQ ID NO:13; "aterr58":SEQ ID NO:14] isolatable from *Aspergillus terreus* (CBS 220.95) or which DNA sequence is a degenerate variant or equivalent thereof.

"Fragments" and "standard conditions" have the meaning as given above. "Degenerate variant" means in this context a DNA sequence which because of the degeneracy of the genetic code has a different nucleotide sequence as the one referred to but codes for a polypeptide with the same amino acid
15 sequence. "Equivalent" refers in this context to a DNA sequence which codes for polypeptides having phytase activity with an amino acid sequence which differs by deletion, substitution and/or addition of one or more amino acids, preferably up to 50, more preferably up to 20, even more preferably up to 10 or most preferably 5, 4, 3 or 2, from the amino acid sequence of the polypeptide encoded by the DNA sequence to which the equivalent sequence refers to. Amino acid substitutions which do not generally alter the specific
20 activity are known in the state of the art and are described, for example, by H. Neurath and R.L. Hill in "The Proteins" (Academic Press, New York, 1979, see especially Figure 6, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse (the three letter abbreviations are used for amino acids and are standard and known in the art).

25 Such equivalents can be produced by methods known in the state of the art and described, e.g. in Sambrook et al. (s.a.). Whether polypeptides encoded by such equivalent sequences still have a phytase activity can be determined by one of the assays known in the art or, e.g. described in Example 9.

It is also an object of the present invention to provide one of the aforementioned DNA sequences which code for a polypeptide having phytase activity which DNA sequence is derived from a fungus, or more
30 specifically such a fungus selected from one of the above mentioned specific groups of fungi.

Furthermore it is an object of the present invention to provide a DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence hybridizes under standard conditions with a probe which is a product of a PCR reaction with DNA isolated from a fungus of one of the above mentioned groups of fungi and the following pair of PCR primer:

35 "ATGGA(C/T)ATGTG(C/T)TC(N)TT(C/T)GA" [SEQ ID NO:15] as sense primer and
"TT(A/G)CC(A/G)GC(A/G)CC(G/A)TG(N)CC(A/G)TA" [SEQ ID NO: 16] as anti-sense primer.

"Standard conditions" have the meaning given above. "Product of a PCR reaction" means preferably a product obtainable or more preferably as obtained by a reaction described in Example 12 referring back to Example 11.

40 Furthermore it is an object of the present invention to provide a DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence hybridizes under standard conditions with a probe which is a product of a PCR reaction with DNA isolated from *Aspergillus terreus* (CBS 220.95) and the following two pairs of PCR primers:

(a) "ATGGA(C/T)ATGTG(C/T)TC(N)TT(C/T)GA" [SEQ ID NO:15] as the sense primer and
45 "TT(A/G)CC(A/G)GC(A/G)CC(G/A)TG(N)CC(A/G)TA" [SEQ ID NO:16] as the anti-sense primer; and
(b) "TA(C/T)GC(N)GA(C/T)TT(C/T)TC(N)CA(C/T)GA" [SEQ ID NO: 17] as the sense primer and
"CG(G/A)TC(G/A)TT(N)AC(N)AG(N)AC(N)C" [SEQ ID NO: 18] as the anti-sense primer.

"Standard conditions" are as defined above and the term "product of a PCR reaction" means preferably a product obtainable or more preferably as obtained by a reaction described in Example 11.

50 It is furthermore an object of the present invention to provide a DNA sequence coding for a chimeric construct having phytase activity which chimeric construct comprises a fragment of a DNA sequence as specified above or preferably such a DNA sequence wherein the chimeric construct consists at its N-terminal end of a fragment of the *Aspergillus niger* phytase fused at its C-terminal end to a fragment of the *Aspergillus terreus* phytase, or more preferably such a DNA sequence with the specific nucleotide
55 sequence as shown in Figure 7 [SEQ ID NO:11] and a degenerate variant or equivalent thereof, wherein "degenerate variant" and "equivalent" have the meanings as given above.

Furthermore it is an object of the present invention to provide a DNA sequence as specified above wherein the encoded polypeptide is a phytase.

Genomic DNA or cDNA from fungal strains can be prepared as known in the art [see e.g. Yelton et al., *Procd. Natl. Acad. Sci. USA*, 1470-1474 (1984) or Sambrook et al., s.a., or, e.g. as specifically described in Example 2.

The cloning of the DNA-sequences of the present invention from such genomic DNA can than be effected, e.g. by using the well known polymerase chain reaction (PCR) method. The principles of this method are outlined e.g. by White et al. (1989), whereas improved methods are described e.g. in Innis et al. [PCR Protocols: A guide to Methods and Applications, Academic Press, Inc. (1990)]. PCR is an in vitro method for producing large amounts of a specific DNA of defined length and sequence from a mixture of different DNA-sequences. Thereby, PCR is based on the enzymatic amplification of the specific DNA fragment of interest which is flanked by two oligonucleotide primers which are specific for this sequence and which hybridize to the opposite strand of the target sequence. The primers are oriented with their 3' ends pointing toward each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment between the PCR primers. Since the extension product of each primer can serve as a template for the other, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. By utilizing the thermostable Taq DNA polymerase, isolated from the thermophilic bacteria *Thermus aquaticus*, it has been possible to avoid denaturation of the polymerase which necessitated the addition of enzyme after each heat denaturation step. This development has led to the automation of PCR by a variety of simple temperature-cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension. The increased specificity improves the overall yield of amplified products by minimizing the competition by non-target fragments for enzyme and primers. In this way the specific sequence of interest is highly amplified and can be easily separated from the non-specific sequences by methods known in the art, e.g. by separation on an agarose gel and cloned by methods known in the art using vectors as described e.g. by Holten and Graham in *Nucleic Acid Res.* 19, 1156 (1991), Kovalic et. al. in *Nucleic Acid Res.* 19, 4560 (1991), Marchuk et al. in *Nucleic Acid Res.* 19, 1154 (1991) or Mead et al. in *Bio/Technology* 9, 657-663 (1991).

The oligonucleotide primers used in the PCR procedure can be prepared as known in the art and described e.g. in Sambrook et al. (1989 "Molecular cloning" 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor).

The specific primers used in the practice of the present invention have been designed as degenerate primers on the basis of DNA-sequence comparisons of known sequences of the *Aspergillus niger* phytase, the *Aspergillus niger* acid phosphatase, the *Saccharomyces cerevisiae* acid phosphatase and the *Schizosaccharomyces pombe* acid phosphatase (for sequence information see, e.g. European Bioinformatics Institute (Hinxton Hall, Cambridge, GB). The degeneracy of the primers was reduced by selecting some codons according to a codon usage table of *Aspergillus niger* prepared on the basis of known sequences from *Aspergillus niger*. Furthermore it has been found that the amino acid at the C-terminal end of the amino acid sequences used to define the specific probes should be a conserved amino acid in all acid phosphatases including phytases specified above but the rest of the amino acids should be more phytase than phosphatase specific.

Such amplified DNA-sequences can than be used to screen DNA libraries of DNA of, e.g. fungal origin by methods known in the art (Sambrook et al., s.a.) or as specifically described in Examples 5-7.

Once complete DNA-sequences of the present invention have been obtained they can be integrated into vectors by methods known in the art and described e.g. in Sambrook et al. (s.a.) to overexpress the encoded polypeptide in appropriate host systems. However, a man skilled in the art knows that also the DNA-sequences themselves can be used to transform the suitable host systems of the invention to get overexpression of the encoded polypeptide. Appropriate host systems are for example fungi, like *Aspergilli*, e.g. *Aspergillus niger* [ATCC 9142] or *Aspergillus ficuum* [NRRL 3135] or like *Trichoderma*, e.g. *Trichoderma reesei* or yeasts, like *Saccharomyces*, e.g. *Saccharomyces cerevisiae* or *Pichia*, like *Pichia pastoris*, all available from ATCC. Bacteria which can be used are e.g. *E. coli*, *Bacilli* as, e.g. *Bacillus subtilis* or *Streptomyces*, e.g. *Streptomyces lividans* (see e.g. Anné and Mallaert in *FEMS Microbiol. Letters* 114, 121 (1993). *E. coli*, which could be used are *E. coli* K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in *J. Bacteriol.* 120, 466-474 (1974)], HB 101 [ATCC No. 33694] or *E. coli* SG13009 [Gottesman et al., *J. Bacteriol.* 148, 265-273 (1981)].

Vectors which can be used for expression in fungi are known in the art and described e.g. in EP 420 358, or by Cullen et al. [*Bio/Technology* 5, 369-376 (1987)] or Ward in *Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi*, Marcel Dekker, New York (1991), Upshall et al. [*Bio/Technology* 5, 1301-1304 (1987)] Gwynne et al. [*Bio/Technology* 5, 71-79 (1987)], Punt et al. [*J. of*

Biotechnology 17, 19-34 (1991)) and for yeast by Sreekrishna et al. [J. Basic Microbiol. 28, 265-278 (1988), Biochem. 28, 4117-4125 (1989)], Hitzemann et al. [Nature 293, 717-722 (1981)] or in EP 183 070, EP 183 071, EP 248 227, EP 263 311. Suitable vectors which can be used for expression in *E. coli* are mentioned, e.g. by Sambrook et al. [s.a.] or by Fiers et al. in Proc. 8th Int. Biotechnology Symposium" [Soc. Franc. de Microbiol., Paris (Durand et al., eds.), pp. 680-697 (1988)] or by Bujard et al. in Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc. Vol. 155, 416-433 (1987) and Stüber et al. in Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152 (1990). Vectors which could be used for expression in *Bacilli* are known in the art and described, e.g. in EP 405 370, Proc. Nat. Acad. Sci. USA 81, 439 (1984) by Yansura and Henner, Meth. Enzym. 185, 199-228 (1990) or EP 207 459.

10 Either such vectors already carry regulatory elements, e.g. promoters or the DNA-sequences of the present invention can be engineered to contain such elements. Suitable promotor-elements which can be used are known in the art and are, e.g. for *Trichoderma reesei* the *cbh1*- [Haarki et al., Biotechnology 7, 596-600 (1989)] or the *pki1*-promotor [Schindler et al., Gene 130, 271-275 (1993)], for *Aspergillus oryzae* the *amy*-promotor [Christensen et al., Abstr. 19th Lunteren Lectures on Molecular Genetics F23 (1987), Christensen et al., Biotechnology 6, 1419-1422 (1988), Tada et al., Mol. Gen. Genet. 229, 301 (1991)], for *Aspergillus niger* the *glaA*- [Cullen et al., Bio/Technology 5, 369-376 (1987), Gwynne et al., Bio/Technology 5, 713-719 (1987), Ward in Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Marcel Dekker, New York, 83-106 (1991)], *alcA*- [Gwynne et al., Bio/Technology 5, 71-719 (1987)], *suc1*- [Boddy et al. Current Genetics 24, 60-66 (1993)], *aphA*- [MacRae et al., Gene 71, 339-348 (1988), MacRae et al., Gene 132, 193-198 (1993)], *tpiA*- [McKnight et al., Cell 46, 143-147 (1986), Upshall et al., Bio/Technology 5, 1301-1304 (1987)], *gpdA*- [Punt et al., Gene 69, 49-57 (1988), Punt et al., J. of Biotechnology 17, 19-37 (1991)] and the *pkiA*-promotor [de Graaff et al., Curr. Genet. 22, 21-27 (1992)]. Suitable promotor-elements which could be used for expression in yeast are known in the art and are, e.g. the *pho5*-promotor [Vogel et al., Molecular and Cellular Biology, 2050-2057 (1989); Rudolf and Hinnen, Proc. Natl. Acad. Sci. 84, 1340-1344 (1987)] or the *gap*-promotor for expression in *Saccharomyces cerevisiae* und for *Pichia pastoris*, e.g. the *aox1*-promotor [Koutz et al. Yeast 5, 167-177 (1989); Sreekrishna et al., J. Basic Microbiol. 28, 265-278 (1988)].

Accordingly vectors comprising DNA sequences of the present invention, preferably for the expression of said DNA sequences in bacteria or a fungal or a yeast host and such transformed bacteria or fungal or yeast hosts are also an object of the present invention.

Once such DNA-sequences have been expressed in an appropriate host cell in a suitable medium the encoded phytase can be isolated either from the medium in the case the phytase is secreted into the medium or from the host organism in case such phytase is present intracellularly by methods known in the art of protein purification or described, e.g. in EP 420 358. Accordingly a process for the preparation of a polypeptide of the present invention characterized in that transformed bacteria or a host cell as described above is cultured under suitable culture conditions and the polypeptide is recovered therefrom and a polypeptide when produced by such a process or a polypeptide encoded by a DNA sequence of the present invention are also an object of the present invention.

Once obtained the polypeptides of the present invention can be characterized regarding their activity by assays known in the state of the art or as described, e.g. by Engelen et al. [J. AOAC Intern. 77, 760-764 (1994)] or in Example 9. Regarding their properties which make the polypeptides of the present invention useful in agriculture any assay known in the art and described e.g. by Simons et al. [British Journal of Nutrition 64, 525-540 (1990)], Schöner et al. [J. Anim. Physiol. a. Anim. Nutr. 66, 248-255 (1991)], Vogt [Arch. Geflügelk. 56, 93-98 (1992)], Jongbloed et al. [J. Anim. Sci., 70, 1159-1168 (1992)], Perney et al. [Poultry Science 72, 2106-2114 (1993)], Farrell et al., [J. Anim. Physiol. a. Anim. Nutr. 69, 278-283 (1993), Broz et al., [British Poultry Science 35, 273-280 (1994)] and Düngethoef et al. [Animal Feed Science and Technology 49, 1-10 (1994)] can be used. Regarding their thermotolerance any assay known in the state of the art and described, e.g. by Yamada et al. (s.a.), and regarding their pH and substrate specificity profiles any assays known in the state of the art and described, e.g. in Example 9 or by Yamada et al., s.a., can be used.

In general the polypeptides of the present invention can be used without being limited to a specific field of application for the conversion of phytate to inositol and inorganic phosphate.

Furthermore the polypeptides of the present invention can be used in a process for the preparation of compound food or feeds wherein the components of such a composition are mixed with one or more polypeptides of the present invention. Accordingly compound food or feeds comprising one or more polypeptides of the present invention are also an object of the present invention. A man skilled in the art is familiar with their process of preparation. Such compound foods or feeds can further comprise additives or components generally used for such purpose and known in the state of the art.

It is furthermore an object of the present invention to provide a process for the reduction of levels of phytate in animal manure characterized in that an animal is fed such a feed composition in an amount effective in converting phytate contained in the feedstuff to inositol and inorganic phosphate.

5 Examples

Specific media and solutions used

Complete medium (Clutterbuck)

10

Glucose	10 g/l
-CN solution	10 ml/l
Sodium nitrate	6 g/l
Bacto peptone (Difco Lab., Detroit, MI, USA)	2 g/l
Yeast Extract (Difco)	1 g/l
Casamino acids (Difco)	1.5 g/l
Modified trace element solution	1 ml/l
Vitamin solution	1 ml/l

15

20

M3 Medium

25

Glucose	10 g/l
-CN Solution	10 ml/l
Modified trace element solution	1 ml/l
Ammonium nitrate	2 g/l

30

M3 Medium - Phosphate

35 M3 medium except that -CN is replaced with -CNP

M3 Medium - Phosphate + Phytate

40 M3 Medium - Phosphate with the addition of 5 g/l of Na₁₂ Phytate (Sigma #P-3168; Sigma, St. Louis, MO, USA)

Modified trace element solution

45

CuSO ₄	0.04%
FeSO ₄ •7H ₂ O	0.08%
Na ₂ MoO ₄ •2H ₂ O	0.08%
ZnSO ₄ •7H ₂ O	0.8%
B ₄ Na ₂ O ₇ •10H ₂ O	0.004%
MnSO ₄ •H ₂ O	0.08%

50

55

EP 0 684 313 A2

Vitamin Solution

5

10

Riboflavin	0.1%
Nicotinamide	0.1%
p-amino benzoic acid	0.01%
Pyridoxine/HCl	0.05%
Aneurine/HCl	0.05%
Biotin	0.001%

-CN Solution

15

20

KH ₂ PO ₄	140 g/l
K ₂ PO ₄ • 3H ₂ O	90 g/l
KCl	10 g/l
MgSO ₄ • 7H ₂ O	10 g/l

-CNP Solution

25

30

HEPES	47.6g/200 mls
KCl	2 g/200 mls
MgSO ₄ • 7H ₂ O	2 g/200 mls

Example 1

Screening fungi for phytase activity

35

Fungi were screened on a three plate system, using the following three media:

- "M3" (a defined medium containing phosphate),
- "M3-P" (M3 medium lacking phosphate) and
- "M3-P + Phytate" (M3 medium lacking phosphate but containing phytate as a sole phosphorus source).

40

Plates were made with agarose to decrease the background level of phosphate.

Fungi were grown on the medium and at the temperature recommended by the supplier. Either spores or mycelium were transferred to the test plates and incubated at the recommended temperature until growth was observed.

45

The following thermotolerant strains were found to exhibit such growth:

- Myceliophthora thermophila* [ATCC 48 102]
- Talaromyces thermophilus* [ATCC 20 186]
- Aspergillus fumigatus* [ATCC 34 625]

Example 2

Growth of fungi and preparation of genomic DNA

50

Strains of *Myceliophthora thermophila*, *Talaromyces thermophilus*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus terreus* 9A-1, and *Aspergillus terreus* CBS 220.95 were grown in Potato Dextrose Broth (Difco Lab., Detroit, MI, USA) or complete medium (Clutterbuck). *Aspergillus terreus* 9A-1 and *Aspergillus nidulans* have been deposited under the Budapest Treaty for patent purposes at the DSM in Braunschweig, BRD at March 17, 1994 under accession number DSM 9076 and at February 17, 1995 under accession

number DSM 9743, respectively.

Genomic DNA was prepared as follows:

Medium was inoculated at a high density with spores and grown O/N with shaking. This produced a thick culture of small fungal pellets. The mycelium was recovered by filtration blotted dry and weighed. Up to 2.0g was used per preparation. The mycelium was ground to a fine powder in liquid nitrogen and immediately added to 10 mls of extraction buffer (200 mM Tris/HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, pH 8.5) and mixed well. Phenol (7 mls) was added to the slurry and mixed and then chloroform (3 mls) was also added and mixed well. The mixture was centrifuged (20,000 g) and the aqueous phase recovered. RNase A was added to a final concentration of 250 µg/ml and incubated at 37 °C for 15 minutes. The mixture was then extracted with 1 volume of chloroform and centrifuged (10,000 g, 10 minutes). The aqueous phase was recovered and the DNA precipitated with 0.54 volumes of RT isopropanol for 1 hour at RT. The DNA was recovered by spooling and resuspended in water.

The resultant DNA was further purified as follows:

A portion of the DNA was digested with proteinase K for 2 hrs at 37 °C and then extracted repeatedly (twice to three times) with an equal volume of phenol/chloroform and then ethanol precipitated prior to resuspension in water to a concentration of approximately 1 µg/µl.

Example 3

20 Degenerate PCR

PCR was performed essentially according to the protocol of Perkin Elmer Cetus [(PEC); Norwalk, CT, USA]. The following two primers were used (bases indicated in brackets are either/or):

Phyt 8: 5' ATG GA(CT) ATG TG(CT) TCN TT(CT) GA 3' [SEQ ID NO:19] Degeneracy = 32
Tm High = 60 °C/ Tm Low 52 °C
Phyt 9: 5' TT(AG) CC(AG) GC(AG) CC(GA) TGN CC(GA) TA 3' [SEQ ID NO:20]
Tm High = 70 °C/Tm Low 58 °C

A typical reaction was performed as follows:

30	H ₂ O	24.5 µl
	10 X PEC GeneAmp Buffer	5 µl
	GeneAmp dNTP's (10 mM)	8 µl
	Primer 1 (Phyt 8, 100 µM)	5 µl
	Primer 2 (Phyt 9, 100 µM)	5 µl
35	DNA (~1 µg/µl)	1 µl
	Taq Polymerase (PEC)	0.5 µl
		50 µl

40 All components with the exception of the Taq polymerase were incubated at 95 °C for 10 minutes and then 50 °C for 10 minutes and then the reaction placed on ice. The Taq polymerase (Amplitaq, Hoffmann-La Roche, Basel, CH) was then added and 35 cycles of PCR performed in a Triothermoblock (Biometra, Göttingen, DE) according to the following cycle profile:

95 °C/ 60"
45 50 °C/ 90"
72 °C/ 120"

An aliquot of the reaction was analysed on 1.5% agarose gel.

Example 4

50 Subcloning and sequencing of PCR fragments

PCR products of the expected size (approximately 146 bp predicted from the *Aspergillus niger* DNA-sequence) were excised from low melting point agarose and purified from a NACS - PREPAC - column (BRL Life Technologies Inc., Gaithersburg, MD, USA) essentially according to the manufacturer's protocol. The fragment was polyadenylated in 50 µl 100 mM Sodiumcacodylate pH6.6, 12.5 mM Tris/HCl pH 7.0, 0.1 mM Dithiothreitol, 125 µg/ml bovine serum albumin, 1 mM CoCl₂, 20 µM dATP, 10 units terminal deoxytransferase (Boehringer Mannheim, Mannheim, DE) for 5 minutes at 37 °C and cloned into the p123T

vector [Mitchell et al., PCR Meth. App. 2, 81-82 (1992)].

Alternatively, PCR fragments were purified and cloned using the "Sure Clone" ligation kit (Pharmacia) following the manufacturers instructions.

Sequencing was performed on dsDNA purified on a Quiagen-column

- 5 (Diagen GmbH, Hilden, DE) using the dideoxy method and the Pharmacia T7 kit (Pharmacia, LKB Biotechnology AB, Uppsala, SE) according to the protocol supplied by the manufacturer.

Example 5

10 Construction and Screening of Lambda Fix II libraries

The fragments from *Aspergillus terreus* Strain 9A-1 and *Myceliophthora thermophila* were used to probe Bam HI and BglII southern to determine the suitable restriction enzyme to use to construct genomic libraries in the Lambda Fix II vector (Stratagene, La Jolla, CA, USA). Lambda Fix II can only accept inserts
15 from 9-23 kb. Southern were performed according to the following protocol. Genomic DNA (10 µg) was digested in a final volume of 200 µl. The reaction without enzyme was prepared and incubated on ice for 2 hours. The enzyme (50 units) was added and the reaction incubated at the appropriate temperature for 3 hours. The reaction was then extracted with an equal volume of phenol/chloroform and ethanol precipitated. The resuspended DNA in loading buffer was heated to 65 °C for 15 minutes prior to separation on a 0.7%
20 agarose gel (O/N 30 V). Prior to transfer the gel was washed twice in 0.2 M HCl/ 10'/room temperature (RT) and then twice in 1M NaCl/0.4M NaOH for 15' at RT. The DNA was transferred in 0.4M NaOH in a capillary transfer for 4 hours to Nytran 13N nylon membrane (Schleicher and Schuell AG, Feldbach, Zürich, CH). Following transfer the membrane was exposed to UV. [Auto cross-link, UV Stratalinker 2400, Stratagene (La Jolla, CA, USA)].

- 25 The membrane was prehybridized in hybridization buffer [50 % formamide, 1% sodium dodecylsulfate (SDS), 10% dextran sulfate, 4 x SSPE (180 mM NaCl, 10 mM NaH₂ PO₄, 1 mM EDTA, pH 7.4)] for 4 hours at 42 °C and following addition of the denatured probe O/N at 42 °C. The blot was washed:

1 x SSPE/0.5 % SDS/RT/30 minutes

0.1 x SSPE/0.1 % SDS/RT/30 minutes

- 30 0.1 x SSPE/0.1 % SDS/65 °C/30 minutes

Results indicate that *Aspergillus terreus* Strain 9A-1 genomic DNA digested with BamHI and *Myceliophthora thermophila* genomic DNA digested with BglII produce fragments suitable for cloning into the lambda Fix II vector.

The construction of genomic libraries of *Aspergillus terreus* Strain 9A-1 and *Myceliophthora thermophila*
35 in Lambda Fix II was performed according to the manufacturer's protocols (Stratagene).

The lambda libraries were plated out on 10 137 mm plates for each library. The plaques were lifted to Nytran 13N round filters and treated for 1 minute in 0.5 M NaOH/1.5 M NaCl followed by 5 minutes in 0.5 M Tris-HCl pH 8.0/1.5 M NaCl. The filters were then treated in 2 X SSC for 5 minutes and air dried. They were then fixed with UV (1 minute, UV Stratalinker 2400, Stratagene). The filters were hybridized and washed as
40 above. Putative positive plaques were cored and the phage soaked out in SM buffer (180 mM NaCl, 8 mM MgSO₄ · 7H₂O, 20mMTris/HCl pH 7.5, 0.01% gelatin). This stock was diluted and plated out on 137 mm plates. Duplicate filters were lifted and treated as above. A clear single positive plaque from each plate was picked and diluted in SM buffer. Three positive plaques were picked. Two from *Aspergillus terreus* Strain 9A-1 (9A1λ17 and 9A1λ22) and one from *Myceliophthora thermophila* (MTλ27).

45 Example 6

Preparation of Lambda DNA and confirmation of the clones

- 50 Lambda DNA was prepared from the positive plaques. This was done using the "Magic Lambda Prep" system (Promega Corp., Madison, WI, USA) and was according to the manufactures specifications. To confirm the identity of the clones, the lambda DNA was digested with PstI and Sall and the resultant blot probed with the PCR products. In all cases this confirmed the clones as containing sequences complementary to the probe.

55

Example 7Subcloning and sequencing of phytase genes

- 5 DNA from 9A1 λ 17 was digested with PstI and the resultant mixture of fragments ligated into pBluescript II SK+ (Stratagene) cut with PstI and treated with shrimp alkaline phosphatase (United States Biochemical Corp., Cleveland, OH, USA). The ligation was O/N at 16 °C. The ligation mixture was transformed into XL-1 Blue Supercompetent cells (Stratagene) and plated on LB Plates containing 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG), 40 μ g/ml 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (Xgal), 50 μ g/ml
- 10 ampicillin.
- DNA from 9A1 λ 17 was digested with Bgl II and Xba I and the resultant mixture ligated into pBluescript II SK+ digested with BamHI/Xba I. Ligation, transformation and screening were performed as described above.
- DNA from MT λ 27 was digested with Sall and the resultant mixture of fragments ligated into pBluescript II
- 15 SK+ cut with Sall and treated with shrimp alkaline phosphatase. The ligation was O/N at 16 °C. The ligation mixture was transformed into XL-1 Blue Supercompetent cells and plated on LB Plates containing Xgal/IPTG and ampicillin.
- Colonies from the above transformations were picked and "gridded" approximately 75 to a single plate. Following O/N incubation at 37 °C the colonies were lifted to a nylon filter ("Hybond-N", Amersham Corp., Arlington Heights, IL, USA) and the filters treated with 0.5M NaOH for 3 minutes, 1M Tris/HCl pH7.5 twice
- 20 for 1 minute, then 0.5M Tris/HCl pH7.5/1.5 M NaCl for 5 minutes. The filters were air dried and then fixed with UV (2 minutes, UV Stratalinker 2400, Stratagene). The filters were hybridized with the PCR products of Example 5. Positive colonies were selected and DNA prepared. The subclones were sequenced as previously described in Example 4. Sequences determined are shown in Figure 1 (Fig. 1) for the phytase
- 25 from *Aspergillus terreus* strain 9A1 and its encoding DNA sequence, Figure 2 for the phytase from *Myceliophthora thermophila* and its encoding DNA-sequence, Figure 3A shows a restriction map for the DNA of *Aspergillus terreus* (wherein the arrow indicates the coding region, and the strips the regions sequenced in addition to the coding region) and 3B for *M. thermophila*, and Figure 4 for part of the phytase from *Talaromyces thermophilus* and its encoding DNA sequence, Figure 5 for part of the phytase from
- 30 *Aspergillus fumigatus* and its encoding DNA-sequence and Figure 6 for part of the phytase from *Aspergillus nidulans* and its encoding DNA-sequence. The sequences for the parts of the phytases and their encoding DNA-sequences from *Talaromyces thermophilus*, *Aspergillus fumigatus* and *Aspergillus nidulans* were obtained in the same way as described for those of *Aspergillus terreus* strain 9A1 and *Myceliophthora thermophila* in Examples 2-7. Bases are given for both strands in small letters by the typically used one
- 35 letter code abbreviations. Derived amino acid sequences of the phytase are given in capital letters by the typically used one letter code below the corresponding DNA-sequence.

Example 840 Construction of a chimeric construct between *A. niger* and *A. terreus* phytase DNA-sequences

- All constructions were made using standard molecular biological procedures as described by Sambrook et al., (1989) (Molecular cloning, A laboratory Manual, Cold Spring Harbor Laboratory Press, NY).
- The first 146 amino acids (aa) of the *Aspergillus niger* phytase, as described in EP 420 358, were fused to
- 45 the 320 C-terminal aa of the *Aspergillus terreus* 9A1 gene. A NcoI site was introduced at the ATG start codon when the *A. niger* phytase gene was cloned by PCR. The intron found in the *A. niger* phytase was removed by site directed mutagenesis (Bio-Rad kit, Cat Nr 170-3581; Bio-Rad, Richmond, CA, USA) using the following primer (wherein the vertical dash indicates that the sequence to its left hybridizes to the 3' end of the first exon and the sequence to its right hybridizes to the 5' end of the second exon):
- 50 5'-AGTCCGGAGGTGACT[CCAGCTAGGAGATAC-3' [SEQ ID NO:21].
- To construct the chimeric construct of phytases from *A. niger* and *A. terreus* an Eco 47III site was introduced into the *A. niger* coding sequence to aid cloning. PCR with a mutagenic primer (5' CGA TTC GTA gCG CTG GTA G 3') in conjunction with the T3 primer was used to produce a DNA fragment that was
- 55 cleaved with Bam HI and Eco 47III. The Bam HI/Eco 47III fragment was inserted into Bam HI/Eco 47III cut p9A1Pst (Example 7). Figure 7 shows the amino acid sequence of the fusion construct and its encoding DNA-sequence.

Example 9Expression of phytases

5 Construction of expression vectors

For expression of the fusion construct in *A. niger* an expression cassette was chosen where the fusion gene was under control of the inducible *A. niger* glucoamylase (*glaA*) promoter.

For the complete *A. terreus* 9A1 gene, expression cassettes with the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter were made.

All genes used for expression in *A. niger* carried their own signal sequence for secretion.

Construction of vector pFPAN1

15 The *A. niger* glucoamylase (*glaA*) promoter was isolated as a 1960 bp XhoI/ClaI fragment from plasmid pDH33 [Smith et al. (1990), Gene 88: 259-262] and cloned into pBluescriptSK⁺-vector (pBS) [Stratagene, La Jolla, CA, USA] containing the 710 bp BamHI/XbaI fragment of the *A. nidulans trpC* terminator. The plasmid with the cassette was named pGLAC. The fusion gene, as described in Example 8, was put under control of the *A. niger glaA* promoter by ligating the blunt ended NcoI/EcoRI fragment to the blunt ended
20 ClaI site and the EcoRV site of plasmid pGLAC. The correct orientation was verified by restriction enzyme digests. The entire cassette was transferred as a KpnI/XbaI fragment to pUC19 (New England Biolabs, GmbH, Schwalbach, BRD), that carried the *Neurospora crassa pyr4* gene (pUC19-*pyr4*), a selection marker in uridine auxotrophic *Aspergilli*, resulting in vector pFPAN1 (see Figure 8 with restriction sites and coding regions as indicated; crossed out restriction sites indicate sites with blunt end ligation).

25

Construction of vector pPAT1

The *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter was isolated as a ~2.3 kb EcoRI/NcoI fragment from plasmid pAN52-1 [Punt et al. (1987), Gene 56: 117-124], cloned into pUC19-NcoI
30 (pUC19 having a SmaI-site replaced by a NcoI-site), reisolated as EcoRI/ BamHI fragment and cloned into pBS with the *trpC* terminator as described above. The obtained cassette was named pGPDN. The *A. terreus* gene was isolated as a NcoI/EcoRI fragment, where the EcoRI site was filled in to create blunt ends. Plasmid pGPDN was cut with BamHI and NcoI. The BamHI site was filled in to create blunt ends. The NcoI/EcoRI(blunt) fragment of the *A. terreus* gene was cloned between the *gpdA* promoter and *trpC*
35 terminator. The expression cassette was isolated as KpnI/XbaI fragment and cloned into pUC19-*pyr4* resulting in plasmid pPAT1 (see Figure 9; for explanation of abbreviations see legend to Figure 8).

Expression of the fusion protein in *Aspergillus niger*

40 A) Transformation

The plasmid pFPAN1 was used to transform *A. niger* by using the transformation protocol as described by Ballance et al. [(1983), Biochem. Biophys. Res. Commun 112, 284-289] with some modifications:

- YPD medium (1 % yeast extract, 2% peptone, 2 % dextrose) was inoculated with 10⁶ spores per ml
45 and grown for 24 hours at 30 ° C and 250 rpm
- cells were harvested using Wero-Lene N tissue (No. 8011.0600 Wernli AG Verbandstoffabrik, 4852 Rothrist, CH) and once washed with buffer (0.8 M KCl, 0.05 M CaCl₂, in 0.01 M succinate buffer; pH 5.5)
- for protoplast preparation only lysing enzymes (SIGMA L-2265, St. Louis, MO, USA) were used
- 50 - the cells were incubated for 90 min at 30 ° C and 100 rpm, and the protoplasts were separated by filtration (Wero-Lene N tissue)
- the protoplasts were once washed with STC (1 M sorbitol, 0.05 M CaCl₂, 0.01 M Tris/HCl pH 7.5) and resuspended in the same buffer
- 150 µl protoplasts (~10⁸/ml) were gently mixed with 10-15 µg plasmid DNA and incubated at room
55 temperature (RT) for 25 min
- polyethylene glycol (60% PEG 4000, 50 mM CaCl₂, 10 mM Tris/HCl pH 7.5) was added in three steps, 150 µl, 200 µl and 900µl, and the sample was further incubated at room temperature (RT) for 25 min

- 5 ml STC were added, centrifuged and the protoplasts were resuspended in 2.5 ml YGS (0.5% yeast extract, 2% glucose, 1.2 M sorbitol)
- the sample was incubated for 2 hours at 30° C (100 rpm) centrifuged and the protoplasts were resuspended in 1 ml 1.2 M sorbitol
- 5 - the transformed protoplasts were mixed with 20 ml minimal regeneration medium (0.7% yeast nitrogen base without amino acids, 2% glucose, 1 M sorbitol, 1.5% agar, 20 mM Tris/HCl pH 7.5 supplemented with 0.2 g arginine and 10 mg nicotinamide per liter)
- the plates were incubated at 30° C for 3-5 days

10 B) Expression

Single transformants were isolated, purified and tested for overproduction of the fusion protein. 100 ml M25 medium (70g maltodextrin (Glucidex 17D, Sugro Basel, CH), 12.5g yeast extract, 25g casein-hydrolysate, 2g KH₂PO₄, 2g K₂SO₄, 0.5g MgSO₄·7H₂O, 0.03g ZnCl₂, 0.02g CaCl₂, 0.05g MnSO₄·4H₂O, 0.05g FeSO₄ 15 per liter pH 5.6) were inoculated with 10⁶ spores per ml from transformants FPAN1#11, #13, #16, #E25, #E30 respectively #E31 and incubated for 5 days at 30° C and 270 rpm. Supernatant was collected and the activity determined. The fusion protein showed the highest activity with phytic acid as substrate at pH 2.5, whereas with 4-nitrophenyl phosphate as substrate it showed two activity optima at pH 2.5 and 5.0 (Table 1).

20

C) Activity assay

a) Phytic acid

A 1 ml enzyme reaction contained 0.5 ml dialyzed supernatant (diluted if necessary) and 5.4 mM phytic acid (SIGMA P-3168). The enzyme reactions were made in 0.2 M sodium acetate buffer pH 5.0, 25 respectively 0.2 M glycine buffer pH 2.5. The samples were incubated for 15 min at 37° C. The reactions were stopped by adding 1 ml 15% TCA (trichloroacetic acid).

For the colour reaction 0.1 ml of the stopped sample was diluted with 0.9 ml distilled water and mixed with 1 ml reagent solution (3 volumes 1 M H₂SO₄, 1 volume 2.5% (NH₄)₆Mo₇O₂₄, 1 volume 10% 30 ascorbic acid). The samples were incubated for 20 min at 50° C and the blue colour was measured spectrophotometrically at 820 nm. Since the assay is based on the release of phosphate a phosphate standard curve, 11 - 45 nmol per ml, was used to determine the activity of the samples.

b) 4-nitrophenyl phosphate

A 1 ml enzyme reaction contained 100 µl dialyzed supernatant (diluted if necessary) and 1.7 mM 4-nitrophenyl phosphate (Merck, 6850, Darmstadt, BRD). The enzyme reactions were made in 0.2 M 35 sodium acetate buffer pH 5.0, respectively 0.2 M glycine buffer pH 2.5. The samples were incubated for 15 min at 37° C. The reactions were stopped by adding 1 ml 15% TCA.

For the determination of the enzyme activity the protocol described above was used.

40

45

50

55

TABLE 1

Transformant	SUBSTRATE			
	* Phytic Acid		* 4-Nitrophenyl phosphate	
	pH 5.0	pH 2.5	pH 5.0	pH 2.5
A. niger ¹⁾	0.2	1	1	2
FPAN1 # 11	6	49	173	399
FPAN1 # 13	2	21	60	228
FPAN1 # 16	1	16	46	153
FPAN1 # E25	3	26	74	228
FPAN1 # E30	3	43	157	347
FPAN1 # E31	3	39	154	271

* Units per ml: 1 unit = 1 μ mol phosphate released per min at 37 ° C

¹⁾ not transformed

Expression of the *Aspergillus terreus* 9A1 gene in *Aspergillus niger*

A. niger NW205 was transformed with plasmid pPAT1 as described above. Single transformants were isolated, purified and screened for overproduction of the *A. terreus* protein. 50 ml YPD medium were inoculated with 10^6 spores per ml from transformants PAT1#3, #10, #11, #13 and #16 and incubated for 3 days at 30 ° C and 270 rpm. Supernatant was collected and the activity determined as described above except that the pH for the enzyme reactions were different. The enzyme showed its main activity at pH 5.5 with phytic acid as substrate and at pH 3.5 with 4-nitrophenyl phosphate as substrate (Table 2).

TABLE 2

Transformant	SUBSTRATE			
	* Phytic Acid		* 4-Nitrophenyl phosphate	
	pH 5.5	pH 3.5	pH 5.5	pH 3.5
A. niger ¹⁾	0	0	0	0.1
PAT1 # 3	10	0	0.2	0.7
PAT1 # 10	9	0	0.2	0.8
PAT1 # 11	5	0	0.1	0.5
PAT1 # 13	9	0	0.2	0.7
PAT1 # 16	5	0	0.1	0.5

* Units per ml: 1 unit = 1 μ mol phosphate released per min at 37 ° C

¹⁾ not transformed

Example 10Fermentation of *Aspergillus niger* NW 205 transformants

5 A) Transformant FPAN1#11

Preculture medium [30 g maltodextrin (Glucidex 17D), 5 g yeast extract, 10 g casein-hydrolysate, 1 g KH_2PO_4 , 0.5g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g Tween 80 per liter; pH 5.5] was inoculated with 10^6 spores per ml in a shake flask and incubated for 24 hours at 34 ° C and 250 rpm.

10 A 10 liter fermenter was inoculated with the pre-culture to a final dilution of the pre-culture of 1:100. The batch fermentation was run at 30 ° C with an automatically controlled dissolved oxygen concentration of minimum 25% ($\text{pO}_2 \geq 25\%$). The pH was kept at 3.0 by automatic titration with 5 M NaOH.

The medium used for the fermentation was: 35 g maltodextrin, 9.4 g yeast extract, 18.7 g casein-hydrolysate, 2 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g K_2SO_4 , 0.03 g ZnCl_2 , 0.02 g CaCl_2 , 0.05 g

15 $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.05 g FeSO_4 per liter; pH 5.6.
Enzyme activities reached after 3 days under these conditions were 35 units/ml respectively 16 units/ml at pH 2.5 respectively pH 5.0 with phytic acid as substrate and 295 units/ml respectively 90 units/ml at pH 2.5 respectively pH 5.0 and 4-nitrophenyl phosphate as substrate.

20 B) Transformant PAT1#11

Preculture, inoculation of the fermenter and the fermentation medium were as described above, except that the pH was kept at 4.5 by automatic titration with 5 M NaOH.

25 Enzyme activities reached after 4 days under these conditions were 17.5 units/ml at pH 5.5 with phytic acid as substrate and 2 units/ml at pH 3.5 with 4-nitrophenyl phosphate as substrate.

Example 11Isolation of PCR fragments of a phytase gene of *Aspergillus terreus* (CBS 220.95)

30 Two different primer pairs were used for PCR amplification of fragments using DNA of *Aspergillus terreus* [CBS 220.95]. The primers used are shown in the Table below.

Fragment amplified	Primers	Oligonucleotide sequences (5' to 3')
35 8 plus 9 about 150 bp	8	ATGGA(C/T)ATGTG(C/T)TC(N)TT(C/T)GA [SEQ ID NO:8]
		Amino acids 254-259: MDMCSF
	9	TT(A/G)CC(A/G)GC(A/G)CC(G/A)TG(N)CC(A/G)TA [SEQ ID NO:9]
		Amino acids 296-301: YGHGAG
40 10 plus 11 about 250 bp	10	TA(C/T)GC(N)GA(C/T)TT(C/T)TC(N)CA(C/T)GA [SEQ ID NO:10]
		Amino acids 349-354: YADFSH
	11	CG(G/A)TC(G/A)TT(N)AC(N)AG(N)AC(N)C [SEQ ID NO:11]
		Amino acids 416-422: RVLVNDNR

50 DNA sequences in bold show the sense primer and in italics the antisense primer. The primers correspond to the indicated part of the coding sequence of the *Aspergillus niger* gene. The combinations used are primers 8 plus 9 and 10 plus 11. The Taq-Start antibody kit from Clontech (Palo Alto, CA, USA) was used according to the manufacturer's protocol. Primer concentrations for 8 plus 9 were 0.2 mM and for primers 10 plus 11 one mM. Touch-down PCR was used for amplification [Don, R.H. et al. (1991), Nucleic Acids Res. 19, 4008]. First the DNA was denatured for 3 min at 95°C. Then two cycles were done at each of the

55 following annealing temperatures: 60°C, 59°C, 58°C, 57°C, 56°C, 55°C, 54°C, 53°C, 52°C and 51°C, with an annealing time of one min. each. Prior to annealing the incubation was heated to 95°C for one min and after annealing elongation was performed for 30 sec at 72°C. Cycles 21 to 35 were performed as follows: denaturation one min at 95°C, annealing one min at 50°C and elongation for 30 sec at 72°C.

Two different PCR fragments were obtained. The DNA sequences obtained and their comparison to relevant parts of the phytase gene of *Aspergillus terreus* 9A1 are shown in Figure 10 [relevant parts of the phytase gene of *Aspergillus terreus* 9A1 "9A1" (top lines) (1) and the PCR fragments of *Aspergillus terreus* CBS 220.95 "aterr21" (bottom lines). Panel A: Fragment obtained with primer pair 8 plus 9 (aterr21). Panel B: Fragment obtained with primer pair 10 plus 11 (aterr58). DNA sequences of *Aspergillus terreus* CBS 220.95 (top lines) are compared with those of *Aspergillus terreus* 9A1 (1) (bottom lines). Panel A: The bold **gc** sequence (bases 16 plus 17) in the aterr21 fragment could possibly be **cg** (DNA sequencing uncertainty). Panel B: The x at position 26 of the aterr58 PCR fragment could possibly represent any of the four nucleotides].

Example 12

Cross hybridizations under non-stringent and stringent washing conditions

- Five μ g's of genomic DNA of each strain listed in Table 3 were incubated with 4 units of *Hind*III or *Pst*I, respectively, per μ g of DNA at 37°C for 4 hours. After digestion, the mixtures were extracted with phenol and DNAs were precipitated with ethanol. Samples were then analyzed on 0.8% agarose gels. DNAs were transferred to Nytran membranes (Schleicher & Schuell, Keene, NH, USA) using 0.4M NaOH containing 1M NaCl as transfer solution. Hybridizations were performed for 18 hours at 42°C. The hybridization solution contained 50% formamide, 1% SDS, 10% dextran sulphate, 4 x SSPE (1 x SSPE = 0.18M NaCl, 1 mM EDTA, 10 mM NaH_2PO_4 , pH 7.4), 0.5% blotto (dried milk powder in H_2O) and 0.5 mg salmon sperm DNA per ml. The membranes were washed under non-stringent conditions using as last and most-stringent washing condition incubation for 30 min at room temperature in 0.1 x SSPE containing 0.1% SDS. The probes (labelled at a specific activity of around 10^9 dpm/ μ g DNA) used were the PCR fragments generated with primers 8 plus 9 (see Example 11) using genomic DNA of *Myceliophthora thermophila*; *Mycelio. thermo.*; *Aspergillus nidulans*, *Asperg. nidul.*; *Aspergillus fumigatus*, *Asperg. fumig.*; *Aspergillus terreus* 9A1, *Asperg. terreus* 9A1. *Talaromyces thermophilus*, *Talarom. thermo.* The **MT2** genomic probe was obtained by random priming (according to the protocol given by Pharmacia, Uppsala, Sweden) and spans 1410 bp, from the BspEI site upstream of the N-terminus of the *Mycelio. thermo.* phytase gene to the PvuII site in the C-terminus (positions 2068 to 3478). The **AT2** genomic probe was obtained by random priming and spans 1365 bp, from the Apal site to the NdeI site of the *Asperg. terreus* 9A1 phytase gene (positions 491 to 1856). The **AN2** DNA probe was obtained by random priming and spans the complete coding sequence (1404 bp) of the *Asperg. niger* gene (EP 420 358). Results are given in Table 3. [""except for weak signal corresponding to a non-specific 20kb fragment; In case of the very weak cross-hybridization signal at 20 kb seen with DNA from *Aspergillus niger* using the PCR fragment from *Talaromyces thermophilus* this signal is unspecific, since it differs significantly from the expected 10 kb *Hind*III fragment, containing the phytase gene; "" signal due to only partial digest of DNA]. For cross-hybridizations with stringent washing conditions membranes were further washed for 30 min. at 65°C in 0.1 x SSPE containing 0.1% SDS. Results are shown in Table 4 [(1) only the 10.5-kb *Hind*III fragment is still detected, the 6.5-kb *Hind*III fragment disappeared (see table 3)].

Table 3

Source of DNA used for cross-hybridization	PCR Probes					Genomic Probes		DNA Probes
	Band (kb) detected with Probe of <i>Asperg.</i> <i>fumig.</i>	Band (kb) detected with Probe of <i>Asperg.</i> <i>nidul.</i>	Band (kb) detected with Probe of <i>Asperg.</i> <i>terreus</i> 9A1	Band (kb) detected with Probe of <i>Mycelio.</i> <i>thermo.</i>	Band (kb) detected with Probe of <i>Talarom.</i> <i>thermo.</i>	Band (kb) detected with geno- mic Probe MT2 of <i>Mycelio.</i> <i>thermo.</i>	Band (kb) detected with geno- mic Probe AT2 of <i>Asperg.</i> <i>terreus</i> 9A1	Band (kb) detected with cDNA Probe AN2 of <i>Asperg.</i> <i>niger</i> (control)
<i>Acrophialophora</i> <i>levis</i> [ATCC 48380]	no	no	no	no	no	8-kb	no	no
<i>Aspergillus niger</i> [ATCC 9142] (control)	no	no	no	no	no*	no	no	10 kb <i>HindIII</i>
<i>Aspergillus terreus</i> [CBS 220.95]	no	no	11-kb <i>HindIII</i>	no	no	no	11-kb <i>HindIII</i>	no
<i>Aspergillus sojae</i> [CBS 221.95]	no	no	no	no	no*	no	3.7-kb <i>HindIII</i>	no
<i>Calcarisporiella</i> <i>thermophila</i> [ATCC 22718]	no	no	10.5-kb <i>HindIII</i>	no	no	10.5-kb <i>HindIII</i>	10.5-kb <i>HindIII</i>	no
<i>Chaetomium</i> <i>rectopilum</i> [ATCC 22431]	no	no	no	no	no	>20-kb** <i>HindIII</i>	>20-kb** <i>HindIII</i>	no
<i>Corynascus</i> <i>thermophilus</i> [ATCC 22066]	no	no	no	no	no	10.5-kb <i>HindIII</i>	no	no
<i>Humicola</i> sp. [ATCC 60849]	no	no	no	no	no	9.5-kb <i>HindIII</i>	no	no
<i>Mycelia sterilia</i> [ATCC 20350]	no	no	no	6-kb <i>HindIII</i>	no	6-kb <i>HindIII</i>	6-kb <i>HindIII</i>	no

	<i>Myrococcum thermophilum</i> [ATCC 22112]	no	no	no	no	4.8-kb <i>Hind</i> III	no	no	no
5	<i>Rhizomucor miehei</i> [ATCC 22064]	no	3.8-kb <i>Hind</i> III	no	no	no	no	no	no
10	<i>Sporotrichum cellulophilum</i> [ATCC 20494]	no	no	no	6-kb <i>Hind</i> III 2.1/3.7- kb <i>Pst</i> I	no	6-kb and 10.5-kb <i>Hind</i> III	6-kb and 10.5-kb <i>Hind</i> III	no
15	<i>Sporotrichum thermophile</i> [ATCC 22482]	no	no	no	6-kb <i>Hind</i> III 2.1/3.7- kb <i>Pst</i> I	6-kb <i>Hind</i> III	6-kb <i>Hind</i> III	6-kb <i>Hind</i> III	no
20	<i>Scytalidium indonesicum</i> [ATCC 46858]	no	no	no	no	no	9-kb <i>Hind</i> III	no	no
	<i>Aspergillus fumigatus</i> [ATCC 34625]	2.3-kb <i>Hind</i> III	no	no	no	no	no	no	no
25	<i>Aspergillus nidulans</i> [DSM 9743]	no	9.5-kb <i>Hind</i> III	no	no	no	no	9.5-kb <i>Hind</i> III	no
30	<i>Aspergillus terreus</i> 9A1 [DSM 9076]	no	no	10.5-kb <i>Hind</i> III	no	6.5-kb <i>Hind</i> III	10.5-kb <i>Hind</i> III	10.5-kb <i>Hind</i> III	no
	<i>Myceliophthora thermophila</i> [ATCC 48102]	no	no	no	6.5-kb <i>Hind</i> III	no	6.5-kb <i>Hind</i> III	6.5-kb <i>Hind</i> III	no
35	<i>Talaromyces thermophilus</i> [ATCC 20186]	no	no	no	no	9.5-kb <i>Hind</i> III	no	no	no

Table 4

Source of DNA used for cross-hybridization	Probe <i>Asperg. fumig.</i>	Probe <i>Asperg. nidul.</i>	Probe <i>Asperg. terreus</i> 9A1	Probe <i>Mycelio. thermo.</i>	Probe <i>Talarom. thermo.</i>	Genomic Probe of MT2 <i>Mycelio. thermo.</i>	Genomic Probe of AT2 <i>Asperg. terreus</i> 9A1	DNA Probe of AN2 <i>Asperg. niger</i> (control)
<i>Acrophialophora levis</i>						yes		
<i>Aspergillus niger</i> (control)								yes
<i>Aspergillus terreus</i> (CBS 116.46)			yes				yes	
<i>Calcarisporiella thermophila</i>			yes				yes	
<i>Chaetomium rectopilum</i>						yes		
<i>Corynascus thermophilus</i>						yes		
<i>Sporotrichum cellulophilum</i>				yes		yes	yes ⁽¹⁾	
<i>Sporotrichum thermophile</i>				yes		yes		
<i>Aspergillus fumigatus</i>	yes							
<i>Aspergillus nidulans</i>		yes						
<i>Aspergillus terreus</i> 9A1			yes				yes	
<i>Mycelia sterilia</i>						yes		
<i>Myceliophthora thermophila</i>				yes		yes		
<i>Talaromyces thermophilus</i>					yes			

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT:
(A) NAME: F. HOFFMANN-LA ROCHE AG
(B) STREET: Grenzacherstrasse 124
(C) CITY: Basle
(D) STATE: BS
(E) COUNTRY: Switzerland
10 (F) POSTAL CODE (ZIP): CH-4002
(G) TELEPHONE: 061 - 688 25 05
(H) TELEFAX: 061 - 688 13 95
(I) TELEX: 962292/965542 hlr ch
- (ii) TITLE OF INVENTION: Polypeptides with phytase activity
15 (iii) NUMBER OF SEQUENCES: 21
- (iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
20 (C) OPERATING SYSTEM: System 7.1 (Macintosh)
(D) SOFTWARE: Word 5.0
- (vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: EP 94810228.0
25 (B) FILING DATE: 25-APR-1994

(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2327 base pairs
30 (B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 35 (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(374..420, 469..1819)
- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
- | | |
|--|-----|
| TCTAGAACAA TAACAGGTAC TCCCTAGGTA CCCGAAGGAC CTTGTGGAAA ATGTATGGAG | 60 |
| GTGGACACGG CACCAACCAC CACCCGCGAT GGCGCACGTG GTGCCCTAAC CCCTTGCTCC | 120 |
| 45 CTCAGGATGG AATCCATGTC GACTCTTTAC CCTCACCATC GCCTGGATGA AACCTCCCCG | 180 |
| CTAAGCTCAC GACGATCGCT ATTTCCGACC GATTTGACCG TCATGGTGGA GGGCTGATTC | 240 |
| GGTCGATGCT CCTGCCTTCA TTTCGGAGTT CGGAGACATG AAAGGCTTAT ATGAGGACGT | 300 |
| 50 CCCAGGTCGG GGACGAAATC CGCCCTGGGC TGTGCTCCTT CGTCGGAAAC ATCTGCTGTC | 360 |
- 55

EP 0 684 313 A2

	CGTGATGGCT ACC ATG GGC TTT CTT GCC ATT GTG CTC TCC GTC GCC TTG	409
	Met Gly Phe Leu Ala Ile Val Leu Ser Val Ala Leu	
	1 5 10	
5	CTC TTT AGA AG GTATGCACCC CTCTACGTCC AATTCTCTGG GCACTGACAA	460
	Leu Phe Arg Ser	
	15	
	CGGCGCAG C ACA TCG GGC ACC CCG TTG GGC CCC CGG GGC AAA CAT AGC	508
10	Thr Ser Gly Thr Pro Leu Gly Pro Arg Gly Lys His Ser	
	20 25	
	GAC TGC AAC TCA GTC GAT CAC GGC TAT CAA TGC TTT CCT GAA CTC TCT	556
	Asp Cys Asn Ser Val Asp His Gly Tyr Gln Cys Phe Pro Glu Leu Ser	
	30 35 40 45	
15	CAT AAA TGG GGA CTC TAC GCG CCC TAC TTC TCC CTC CAG GAC GAG TCT	604
	His Lys Trp Gly Leu Tyr Ala Pro Tyr Phe Ser Leu Gln Asp Glu Ser	
	50 55 60	
	CCG TTT CCT CTG GAC GTC CCA GAG GAC TGT CAC ATC ACC TTC GTG CAG	652
20	Pro Phe Pro Leu Asp Val Pro Glu Asp Cys His Ile Thr Phe Val Gln	
	65 70 75	
	GTG CTG GCC CGC CAC GGC GCG CGG AGC CCA ACC CAT AGC AAG ACC AAG	700
	Val Leu Ala Arg His Gly Ala Arg Ser Pro Thr His Ser Lys Thr Lys	
	80 85 90	
25	GCG TAC GCG GCG ACC ATT GCG GCC ATC CAG AAG AGT GCC ACT GCG TTT	748
	Ala Tyr Ala Ala Thr Ile Ala Ala Ile Gln Lys Ser Ala Thr Ala Phe	
	95 100 105	
	CCG GGC AAA TAC GCG TTC CTG CAG TCA TAT AAC TAC TCC TTG GAC TCT	796
30	Pro Gly Lys Tyr Ala Phe Leu Gln Ser Tyr Asn Tyr Ser Leu Asp Ser	
	110 115 120 125	
	GAG GAG CTG ACT CCC TTC GGG CGG AAC CAG CTG CGA GAT CTG GGC GCC	844
	Glu Glu Leu Thr Pro Phe Gly Arg Asn Gln Leu Arg Asp Leu Gly Ala	
	130 135 140	
35	CAG TTC TAC GAG CGC TAC AAC GCC CTC ACC CGA CAC ATC AAC CCC TTC	892
	Gln Phe Tyr Glu Arg Tyr Asn Ala Leu Thr Arg His Ile Asn Pro Phe	
	145 150 155	
	GTC CGC GCC ACC GAT GCA TCC CGC GTC CAC GAA TCC GCC GAG AAG TTC	940
40	Val Arg Ala Thr Asp Ala Ser Arg Val His Glu Ser Ala Glu Lys Phe	
	160 165 170	
	GTC GAG GGC TTC CAA ACC GCT CGA CAG GAC GAT CAT CAC GCC AAT CCC	988
	Val Glu Gly Phe Gln Thr Ala Arg Gln Asp Asp His His Ala Asn Pro	
	175 180 185	
45	CAC CAG CCT TCG CCT CGC GTG GAC GTG GCC ATC CCC GAA GGC AGC GCC	1036
	His Gln Pro Ser Pro Arg Val Asp Val Ala Ile Pro Glu Gly Ser Ala	
	190 195 200 205	
	TAC AAC AAC ACG CTG GAG CAC AGC CTC TGC ACC GCC TTC GAA TCC AGC	1084
50	Tyr Asn Asn Thr Leu Glu His Ser Leu Cys Thr Ala Phe Glu Ser Ser	
	210 215 220	

55

EP 0 684 313 A2

	ACC	GTC	GGC	GAC	GAC	GCG	GTC	GCC	AAC	TTC	ACC	GCC	GTG	TTC	GCG	CCG	1132
	Thr	Val	Gly	Asp	Asp	Ala	Val	Ala	Asn	Phe	Thr	Ala	Val	Phe	Ala	Pro	
			225						230					235			
5	GCG	ATC	GCC	CAG	CGC	CTG	GAG	GCC	GAT	CTT	CCC	GGC	GTG	CAG	CTG	TCC	1180
	Ala	Ile	Ala	Gln	Arg	Leu	Glu	Ala	Asp	Leu	Pro	Gly	Val	Gln	Leu	Ser	
			240					245					250				
	ACC	GAC	GAC	GTG	GTC	AAC	CTG	ATG	GCC	ATG	TGT	CCG	TTC	GAG	ACG	GTC	1228
10	Thr	Asp	Asp	Val	Val	Asn	Leu	Met	Ala	Met	Cys	Pro	Phe	Glu	Thr	Val	
		255					260					265					
	AGC	CTG	ACC	GAC	GAC	GCG	CAC	ACG	CTG	TCG	CCG	TTC	TGC	GAC	CTC	TTC	1276
	Ser	Leu	Thr	Asp	Asp	Ala	His	Thr	Leu	Ser	Pro	Phe	Cys	Asp	Leu	Phe	
		270				275					280					285	
15	ACG	GCC	ACT	GAG	TGG	ACG	CAG	TAC	AAC	TAC	CTG	CTC	TCG	CTG	GAC	AAG	1324
	Thr	Ala	Thr	Glu	Trp	Thr	Gln	Tyr	Asn	Tyr	Leu	Leu	Ser	Leu	Asp	Lys	
					290					295					300		
	TAC	TAC	GGC	TAC	GGC	GGG	GGC	AAT	CCG	CTG	GGT	CCG	GTG	CAG	GGG	GTC	1372
20	Tyr	Tyr	Gly	Tyr	Gly	Gly	Gly	Asn	Pro	Leu	Gly	Pro	Val	Gln	Gly	Val	
				305					310					315			
	GGC	TGG	GCG	AAC	GAG	CTG	ATG	GCG	CGG	CTA	ACG	CGC	GCC	CCC	GTG	CAC	1420
	Gly	Trp	Ala	Asn	Glu	Leu	Met	Ala	Arg	Leu	Thr	Arg	Ala	Pro	Val	His	
			320					325					330				
25	GAC	CAC	ACC	TGC	GTC	AAC	AAC	ACC	CTC	GAC	GCG	AGT	CCG	GCC	ACC	TTC	1468
	Asp	His	Thr	Cys	Val	Asn	Asn	Thr	Leu	Asp	Ala	Ser	Pro	Ala	Thr	Phe	
		335					340					345					
	CCG	CTG	AAC	GCC	ACC	CTC	TAC	GCC	GAC	TTC	TCC	CAC	GAC	AGC	AAC	CTG	1516
30	Pro	Leu	Asn	Ala	Thr	Leu	Tyr	Ala	Asp	Phe	Ser	His	Asp	Ser	Asn	Leu	
						355					360					365	
	GTG	TCG	ATC	TTC	TGG	GCG	CTG	GGC	CTG	TAC	AAC	GGC	ACC	GCG	CCG	CTG	1564
	Val	Ser	Ile	Phe		Ala	Leu	Gly	Leu	Tyr	Asn	Gly	Thr	Ala	Pro	Leu	
					370					375					380		
35	TCG	CAG	ACC	TCC	GTC	GAG	AGC	GTC	TCC	CAG	ACG	GAC	GGG	TAC	GCC	GCC	1612
	Ser	Gln	Thr		Ser	Val	Glu	Ser	Val	Ser	Gln	Thr	Asp	Gly	Tyr	Ala	
				385					390						395		
	GCC	TGG	ACG	GTG	CCG	TTC	GCC	GCT	CGC	GCG	TAC	GTC	GAG	ATG	ATG	CAG	1660
40	Ala	Trp		Thr	Val	Pro	Phe	Ala	Ala	Arg	Ala	Tyr	Val	Glu	Met	Gln	
			400					405					410				
	TGT	CGC	GCC	GAG	AAG	GAG	CCG	CTG	GTG	CGC	GTG	CTG	GTC	AAC	GAC	CGG	1708
	Cys	Arg	Ala	Glu	Lys	Glu	Pro	Leu	Val	Arg	Val	Leu	Val	Asn	Asp	Arg	
		415					420					425					
45	GTC	ATG	CCG	CTG	CAT	GGC	TGC	CCT	ACG	GAC	AAG	CTG	GGG	CGG	TGC	AAG	1756
	Val	Met	Pro	Leu	His	Gly	Cys	Pro	Thr	Asp	Lys	Leu	Gly	Arg	Cys	Lys	
		430				435					440					445	
	CGG	GAC	GCT	TTC	GTC	GCG	GGG	CTG	AGC	TTT	GCG	CAG	GCG	GGC	GGG	AAC	1804
50	Arg	Asp	Ala	Phe	Val	Ala	Gly	Leu	Ser	Phe	Ala	Gln	Ala	Gly	Gly	Asn	
					450					455					460		

55

TGG GCG GAT TGT TTC TGATGTTGAG AAGAAAGGTA GATAGATAGG TAGTACATAT 1859
 Trp Ala Asp Cys Phe
 465
 5 GGATTGCTCG GCTCTGGGTC GTTGCCACACA ATGCATATTA CGCCCGTCAA CTGCCTTGCG 1919
 CCATCCACCT CTCACCCTGG ACGCAACCGA GCGGTCTACC CTGCACACGG CTTCCACCGC 1979
 GACGCGCACG GATAAGGCGC TTTTGTACG GGGTTGGGGC TGGGGGCAGC CGGAGCCGGA 2039
 10 GAGAGAGACC AGCGTGAAAA ACGACAGAAC ATAGATATCA ATTCGACGCC AATTCATGCA 2099
 GAGTAGTATA CAGACGAAC GAAACAAACA CATCACTTCC CTCGCTCCTC TCCTGTAGAA 2159
 GACGCTCCCA CCAGCCGCTT CTGGCCCTTA TTCCCGTACG CTAGGTAGAC CAGTCAGCCA 2219
 15 GACGCATGCC TCACAAGAAC GGGGGCGGGG GACACACTCC GTCGTACAG CACCCACGAC 2279
 GTGTACAGGA AAACCGGCAG CGCCACAATC GTCGAGAGCC ATCTGCAG 2327

20 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 466 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

30 Met Gly Phe Leu Ala Ile Val Leu Ser Val Ala Leu Leu Phe Arg Ser
 1 5 10 15
 Thr Ser Gly Thr Pro Leu Gly Pro Arg Gly Lys His Ser Asp Cys Asn
 20 25 30
 35 Ser Val Asp His Gly Tyr Gln Cys Phe Pro Glu Leu Ser His Lys Trp
 35 40 45
 Gly Leu Tyr Ala Pro Tyr Phe Ser Leu Gln Asp Glu Ser Pro Phe Pro
 50 55 60
 40 Leu Asp Val Pro Glu Asp Cys His Ile Thr Phe Val Gln Val Leu Ala
 65 70 75 80
 Arg His Gly Ala Arg Ser Pro Thr His Ser Lys Thr Lys Ala Tyr Ala
 85 90 95
 45 Ala Thr Ile Ala Ala Ile Gln Lys Ser Ala Thr Ala Phe Pro Gly Lys
 100 105 110
 Tyr Ala Phe Leu Gln Ser Tyr Asn Tyr Ser Leu Asp Ser Glu Glu Leu
 115 120 125
 50 Thr Pro Phe Gly Arg Asn Gln Leu Arg Asp Leu Gly Ala Gln Phe Tyr
 130 135 140

55

EP 0 684 313 A2

Glu Arg Tyr Asn Ala Leu Thr Arg His Ile Asn Pro Phe Val Arg Ala
 145 150 155 160
 Thr Asp Ala Ser Arg Val His Glu Ser Ala Glu Lys Phe Val Glu Gly
 165 170 175
 Phe Gln Thr Ala Arg Gln Asp Asp His His Ala Asn Pro His Gln Pro
 180 185 190
 Ser Pro Arg Val Asp Val Ala Ile Pro Glu Gly Ser Ala Tyr Asn Asn
 195 200 205
 Thr Leu Glu His Ser Leu Cys Thr Ala Phe Glu Ser Ser Thr Val Gly
 210 215 220
 Asp Asp Ala Val Ala Asn Phe Thr Ala Val Phe Ala Pro Ala Ile Ala
 225 230 235 240
 Gln Arg Leu Glu Ala Asp Leu Pro Gly Val Gln Leu Ser Thr Asp Asp
 245 250 255
 Val Val Asn Leu Met Ala Met Cys Pro Phe Glu Thr Val Ser Leu Thr
 260 265 270
 Asp Asp Ala His Thr Leu Ser Pro Phe Cys Asp Leu Phe Thr Ala Thr
 275 280 285
 Glu Trp Thr Gln Tyr Asn Tyr Leu Leu Ser Leu Asp Lys Tyr Tyr Gly
 290 295 300
 Tyr Gly Gly Gly Asn Pro Leu Gly Pro Val Gln Gly Val Gly Trp Ala
 305 310 315 320
 Asn Glu Leu Met Ala Arg Leu Thr Arg Ala Pro Val His Asp His Thr
 325 330 335
 Cys Val Asn Asn Thr Leu Asp Ala Ser Pro Ala Thr Phe Pro Leu Asn
 340 345 350
 Ala Thr Leu Tyr Ala Asp Phe Ser His Asp Ser Asn Leu Val Ser Ile
 355 360 365
 Phe Trp Ala Leu Gly Leu Tyr Asn Gly Thr Ala Pro Leu Ser Gln Thr
 370 375 380
 Ser Val Glu Ser Val Ser Gln Thr Asp Gly Tyr Ala Ala Ala Trp Thr
 385 390 395 400
 Val Pro Phe Ala Ala Arg Ala Tyr Val Glu Met Met Gln Cys Arg Ala
 405 410 415
 Glu Lys Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Met Pro
 420 425 430
 Leu His Gly Cys Pro Thr Asp Lys Leu Gly Arg Cys Lys Arg Asp Ala
 435 440 445
 Phe Val Ala Gly Leu Ser Phe Ala Gln Ala Gly Gly Asn Trp Ala Asp
 450 455 460

Cys Phe
465

5 (2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3995 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- 15 (A) NAME/KEY: CDS
 (B) LOCATION: join(2208..2263, 2321..3725)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

20 GTCGACGAGG CACACCACGC CCGTCCCTCGG CGGGTCCGAG AGGGCCGGGC TCGGGTTCGA 60
 CAAGGAGACG GGCCTCCCTT CGGGCGCGGC TGCGGGTGTG GGTGTTGCTG TGGACGGTGA 120
 GGAGGGGGAC GGGCTGGGCG TTGATGACGG TACGAATGCG AACGGACACA GGCCGCTGAG 180
 CGTGGGTGTT GCGTTCTAAT CTTTCTTTGT GTGGGTGTGT ACGTGTGGGT GTGTATGTGT 240
 25 TTGGGGGGGG GAATGTTCTT GGTAATTATC TTTCTACCCT TCTTCTCTTT CCTTTATTCT 300
 GTTCAGCAGG TATACCCCGT GTAAGTGTAC AGGATTATGG GACGGGTGGG TGGATGGACT 360
 ACTTCTAGAA GGACGGATAA GGAAAAAGGG GAAACACGAA TATGGCGCCC TGGGTGGCGC 420
 30 GTCGAGCTGG ATGCTTGACG CCGGTCTGGC AAACATTTTC TTCTTCTAGC ACCCAACCTA 480
 GTACTTGATA GAGTGTTCG GGGCCAGGCG GTTTGCGCTG TGTTTTTACC AATCACCAAC 540
 TAGTGCTACT ACTATTATTG CGGCTGTTGA TGCAGCCGTG TACCAAAAAT GCCGCGGCAT 600
 35 CTCCATTGAT ACTTGTAGTT TTGATAGATC AATATTGGG AGGTTGCGCT GGGCTGCTCT 660
 GAAACCCCTC TCTCTTGCTG TACGTAACGT ATGTGCACAG TATGTCACCG ACAAAGACGA 720
 TTGCATGCGC ATCGTTTTTT GTTGTGTTTC AGGCCTCGCT CGTGTCTAGG GTATAAACAC 780
 40 ATTGAAGACT ACATATGCGC AAGACGTTGA CATTACGGG GTCCTGCAGC CGCCGCAGGT 840
 GCATGTCGTG ATTAATACCA CGCGCCTGCG TAAATTAGCT AGCCGCGGCC CTGTTTCACT 900
 CGGTTAGAGA CGGACAGGTG AGACGGGTCT CGGTTAAGCA AGCAAATTGG AATGCAAGGT 960
 45 TGAAGGTGTA ATCTGCATAG CGTGGAAATG AGAGGGCTCT GTGGGCAGCC AGGAAGGTGA 1020
 GACGAAATGA GGAAAGAGGC ACCAGAAGCT GTTGTCTGA AGTGCCCGTG GTCATAGCTC 1080
 CAGGATTAAG TACGGATGTC CCATGCCAAG CTGCTGGCTT CGAAAGCGAG TACGGAGTAG 1140
 50 TGTCCATTGT TCACGAGGGA TCCCAATGT GTTAGACATG CCTGAATCAA TTTTGTCCTA 1200

55

EP 0 684 313 A2

	TTTTTGGATT TCAACTGTTT CTCTCGACTG TGCTCGGTAG CGACTATGCC GCAAGGTACA	1260
	CTACATGTTG TACAATAATC ATACATCGAC CTTCCGTAGG AGTGCTGAAA TACCCGACCT	1320
5	GCTCTCTCTA GCAGGTGCCT AATGGCTTTC GTGTAAC TCG ATCGAAACGG ATCAGCAAGT	1380
	CCATTGCTG TTGGTTGAGA TGTACGATTT ACAAACACGT GGAGAGGTGA GCCACAGCGA	1440
	TAGGCTTCTG GAAGGATTCT GCGTCTCGG AAAGAGGGCC ACTCGCCCCA CTAACCGGCG	1500
10	CCGATCTTGA CATGGGGCTC GCAGGGGGTT TAAGTGCACA CTACGGAGTA CGGATTACAC	1560
	AGTAGTGTAT GGGTGGGGGC GAGTTTGGGT GGCCTTGTGT GGGGCTCACC GGCTGCCTGT	1620
	TCTCGGGGAG TCTTGGCGGG CCGATTGGAC CCACCTAACC ACGGGTAGTC TTGGCCCGGC	1680
15	CAACTCACAC CGCCCTCATG TTTCGGAGCC AGTCAGGGAG GCAGGCACTA CTCAGTCAGG	1740
	TACACACGTC GGGCTCCTCG ATGCTGGGTG ACATCGAGGC GATACTGCAT TCCAAC TACG	1800
	GTTGGCATAG GAGGTATCCT ATTCTAGAGC TGTCTACGC CGGAACGTAA CCCGGGATAA	1860
20	CCCGGGATAT CGCTTCCCTG AGCGAGCGCG CTGCTGAGGA TCATACAACC CAACAACCGA	1920
	CGACGGTGCA AGAAGGTTGG GGAAGGAAG AAATCAAGGA AAAAAAATA GGGGGGGTGG	1980
	GGACCAAGAG AGAAAGAAAG GAGAAAAGGG TGGGGGGAGG GAAGAGAAAA AAAAAACGGA	2040
25	GGAATATGGC GTCGCTCTTC GACTGGTTCC GGAAGGGGGC ATCTGGGTAC ACATATGCAC	2100
	CTCTTCCGCA CGGCAGGGAT ATAAACCGGG AGTGCAGTCC CACCGATCAT GCTGAGTCCG	2160
30	CCCGTCTCCA GACTTCACGG TCGCAGAGGA CTAGACGCGC GGTGAAG ATG ACT GGC Met Thr Gly 1	2216
	CTC GGA GTG ATG GTG GTG ATG GTC GGC TTC CTG GCG ATC GCC TCT CT Leu Gly Val Met Val Val Met Val Gly Phe Leu Ala Ile Ala Ser Leu 5 10 15	2263
35	GTAAGCAGCG ATTCCAGGGG TCCGGTGTGC GTTAAAAGAA AAAGCTAACG CCACCAG A	2321
	CAA TCC GAG TCC CGG CCA TGC GAC ACC CCA GAC TTG GGC TTC CAG TGT Gln Ser Glu Ser Arg Pro Cys Asp Thr Pro Asp Leu Gly Phe Gln Cys 20 25 30 35	2369
40	GGT ACG GCC ATT TCC CAC TTC TGG GGC CAG TAC TCG CCC TAC TTC TCC Gly Thr Ala Ile Ser His Phe Trp Gly Gln Tyr Ser Pro Tyr Phe Ser 40 45 50	2417
	GTG CCC TCG GAG CTG GAT GCT TCG ATC CCC GAC GAC TGC GAG GTG ACG Val Pro Ser Glu Leu Asp Ala Ser Ile Pro Asp Asp Cys Glu Val Thr 55 60 65	2465
45	TTT GCC CAA GTC CTC TCC CGC CAC GGC GCG AGG GCG CCG ACG CTC AAA Phe Ala Gln Val Leu Ser Arg His Gly Ala Arg Ala Pro Thr Leu Lys 70 75 80	2513
50	CGG GCC GCG AGC TAC GTC GAT CTC ATC GAC AGG ATC CAC CAT GGC GCC	2561

55

EP 0 684 313 A2

	Arg	Ala	Ala	Ser	Tyr	Val	Asp	Leu	Ile	Asp	Arg	Ile	His	His	Gly	Ala	
	85						90					95					
5	ATC	TCC	TAC	GGG	CCG	GGC	TAC	GAG	TTC	CTC	AGG	ACG	TAT	GAC	TAC	ACC	2609
	Ile	Ser	Tyr	Gly	Pro	Gly	Tyr	Glu	Phe	Leu	Arg	Thr	Tyr	Asp	Tyr	Thr	
	100					105					110					115	
	CTG	GGC	GCC	GAC	GAG	CTC	ACC	CGG	ACG	GGC	CAG	CAG	CAG	ATG	GTC	AAC	2657
	Leu	Gly	Ala	Asp	Glu	Leu	Thr	Arg	Thr	Gly	Gln	Gln	Gln	Met	Val	Asn	
10					120					125					130		
	TCG	GGC	ATC	AAG	TTT	TAC	CGC	CGC	TAC	CGC	GCT	CTC	GCC	CGC	AAG	TCG	2705
	Ser	Gly	Ile	Lys	Phe	Tyr	Arg	Arg	Tyr	Arg	Ala	Leu	Ala	Arg	Lys	Ser	
				135					140					145			
15	ATC	CCC	TTC	GTC	CGC	ACC	GCC	GGC	CAG	GAC	CGC	GTC	GTC	CAC	TCG	GCC	2753
	Ile	Pro	Phe	Val	Arg	Thr	Ala	Gly	Gln	Asp	Arg	Val	Val	His	Ser	Ala	
				150				155					160				
	GAG	AAC	TTC	ACC	CAG	GGC	TTC	CAC	TCT	GCC	CTG	CTC	GCC	GAC	CGC	GGG	2801
	Glu	Asn	Phe	Thr	Gln	Gly	Phe	His	Ser	Ala	Leu	Leu	Ala	Asp	Arg	Gly	
20				165			170					175					
	TCC	ACC	GTC	CGG	CCC	ACC	CTC	CCC	TAT	GAC	ATG	GTC	GTC	ATC	CCG	GAA	2849
	Ser	Thr	Val	Arg	Pro	Thr	Leu	Pro	Tyr	Asp	Met	Val	Val	Ile	Pro	Glu	
	180					185				190						195	
25	ACC	GCC	GGC	GCC	AAC	AAC	ACG	CTC	CAC	AAC	GAC	CTC	TGC	ACC	GCC	TTC	2897
	Thr	Ala	Gly	Ala	Asn	Asn	Thr	Leu	His	Asn	Asp	Leu	Cys	Thr	Ala	Phe	
					200					205					210		
	GAG	GAA	GGC	CCG	TAC	TCG	ACC	ATC	GGC	GAC	GAC	GCC	CAA	GAC	ACC	TAC	2945
	Glu	Glu	Gly	Pro	Tyr	Ser	Thr	Ile	Gly	Asp	Asp	Ala	Gln	Asp	Thr	Tyr	
30				215				220					225				
	CTC	TCC	ACC	TTC	GCC	GGA	CCC	ATC	ACC	GCC	CGG	GTC	AAC	GCC	AAC	CTG	2993
	Leu	Ser	Thr	Phe	Ala	Gly	Pro	Ile	Thr	Ala	Arg	Val	Asn	Ala	Asn	Leu	
				230				235					240				
35	CCG	GGC	GCC	AAC	CTG	ACC	GAC	GCC	GAC	ACG	GTC	GCG	CTG	ATG	GAC	CTC	3041
	Pro	Gly	Ala	Asn	Leu	Thr	Asp	Ala	Asp	Thr	Val	Ala	Leu	Met	Asp	Leu	
				245			250					255					
	TGC	CCC	TTC	GAG	ACG	GTC	GCC	TCC	TCC	TCC	TCC	GAC	CCG	GCA	ACG	GCG	3089
	Cys	Pro	Phe	Glu	Thr	Val	Ala	Ser	Ser	Ser	Ser	Asp	Pro	Ala	Thr	Ala	
40				260			265				270					275	
	GAC	GCG	GGG	GGC	GGC	AAC	GGG	CGG	CCG	CTG	TCG	CCC	TTC	TGC	CGC	CTG	3137
	Asp	Ala	Gly	Gly	Gly	Asn	Gly	Arg	Pro	Leu	Ser	Pro	Phe	Cys	Arg	Leu	
					280					285					290		
45	TTC	AGC	GAG	TCC	GAG	TGG	CGC	GCG	TAC	GAC	TAC	CTG	CAG	TCG	GTG	GGC	3185
	Phe	Ser	Glu	Ser	Glu	Trp	Arg	Ala	Tyr	Asp	Tyr	Leu	Gln	Ser	Val	Gly	
				295				300					305				
	AAG	TGG	TAC	GGG	TAC	GGG	CCG	GGC	AAC	CCG	CTG	GGG	CCG	ACG	CAG	GGG	3233
	Lys	Trp	Tyr	Gly	Tyr	Gly	Pro	Gly	Asn	Pro	Leu	Gly	Pro	Thr	Gln	Gly	
50				310				315					320				

55

EP 0 684 313 A2

	GTC GGG TTC GTC AAC GAG CTG CTG GCG CGG CTG GCC GGG GTC CCC GTG	3281
	Val Gly Phe Val Asn Glu Leu Leu Ala Arg Leu Ala Gly Val Pro Val	
	325 330 335	
5	CGC GAC GGC ACC AGC ACC AAC CGC ACC CTC GAC GGC GAC CCG CGC ACC	3329
	Arg Asp Gly Thr Ser Thr Asn Arg Thr Leu Asp Gly Asp Pro Arg Thr	
	340 345 350 355	
10	TTC CCG CTC GGC CGG CCC CTC TAC GCC GAC TTC AGC CAC GAC AAC GAC	3377
	Phe Pro Leu Gly Arg Pro Leu Tyr Ala Asp Phe Ser His Asp Asn Asp	
	360 365 370	
	ATG ATG GGC GTC CTC GGC GCC CTC GGC GCC TAC GAC GGC GTC CCG CCC	3425
	Met Met Gly Val Leu Gly Ala Leu Gly Ala Tyr Asp Gly Val Pro Pro	
	375 380 385	
15	CTC GAC AAG ACC GCC CGC CGC GAC CCG GAA GAG CTC GGC GGG TAC GCG	3473
	Leu Asp Lys Thr Ala Arg Arg Asp Pro Glu Glu Leu Gly Gly Tyr Ala	
	390 395 400	
20	GCC AGC TGG GCC GTC CCG TTC GCC GCC AGG ATC TAC GTC GAG AAG ATG	3521
	Ala Ser Trp Ala Val Pro Phe Ala Ala Arg Ile Tyr Val Glu Lys Met	
	405 410 415	
	CGG TGC AGC GGC GGC GGC GGC GGC GGC GGC GGC GAG GGG CGG CAG	3569
	Arg Cys Ser Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Glu Gly Arg Gln	
	420 425 430 435	
25	GAG AAG GAT GAG GAG ATG GTC AGG GTG CTG GTG AAC GAC CGG GTG ATG	3617
	Glu Lys Asp Glu Glu Met Val Arg Val Leu Val Asn Asp Arg Val Met	
	440 445 450	
30	ACG CTG AAG GGG TGC GGC GCC GAC GAG AGG GGG ATG TGT ACG CTA GAA	3665
	Thr Leu Lys Gly Cys Gly Ala Asp Glu Arg Gly Met Cys Thr Leu Glu	
	455 460 465	
	CGG TTC ATC GAA AGC ATG GCG TTT GCG AGG GGG AAC GGC AAG TGG GAT	3713
	Arg Phe Ile Glu Ser Met Ala Phe Ala Arg Gly Asn Gly Lys Trp Asp	
	470 475 480	
35	CTC TGC TTT GCT TGATATGCCC ACGCCCGAGA TTGAACAGAA CTTGTGATGG	3765
	Leu Cys Phe Ala	
	485	
40	GGGTAGAGTG TGGTATTCGA GATGATAGTT CACAGTTTTC GGGAATCAAA AATCGGTTAG	3825
	ACTGGCGAAA TTCAAGTCTG GGGCCTGCGG CGTCTGCATT CTCCGTTCCC TGTTGTTACC	3885
	TTCTTAATGG TTTTTTTTTA TTTTTTATTT TTCTTAAATT TTCACACAAA CCTTTTATTG	3945
45	TCTTTTTTTC TTCTTTTCT TCTTCTGCAC ATCGGATGGG AATTGTCGAC	3995

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 487 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

EP 0 684 313 A2

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```

5   Met Thr Gly Leu Gly Val Met Val Val Met Val Gly Phe Leu Ala Ile
    1           5           10           15

    Ala Ser Leu Gln Ser Glu Ser Arg Pro Cys Asp Thr Pro Asp Leu Gly
      20           25           30

10  Phe Gln Cys Gly Thr Ala Ile Ser His Phe Trp Gly Gln Tyr Ser Pro
    35           40           45

    Tyr Phe Ser Val Pro Ser Glu Leu Asp Ala Ser Ile Pro Asp Asp Cys
    50           55           60

15  Glu Val Thr Phe Ala Gln Val Leu Ser Arg His Gly Ala Arg Ala Pro
    65           70           75           80

    Thr Leu Lys Arg Ala Ala Ser Tyr Val Asp Leu Ile Asp Arg Ile His
    85           90           95

20  His Gly Ala Ile Ser Tyr Gly Pro Gly Tyr Glu Phe Leu Arg Thr Tyr
    100          105          110

    Asp Tyr Thr Leu Gly Ala Asp Glu Leu Thr Arg Thr Gly Gln Gln Gln
    115          120          125

25  Met Val Asn Ser Gly Ile Lys Phe Tyr Arg Arg Tyr Arg Ala Leu Ala
    130          135          140

    Arg Lys Ser Ile Pro Phe Val Arg Thr Ala Gly Gln Asp Arg Val Val
    145          150          155          160

30  His Ser Ala Glu Asn Phe Thr Gln Gly Phe His Ser Ala Leu Leu Ala
    165          170          175

    Asp Arg Gly Ser Thr Val Arg Pro Thr Leu Pro Tyr Asp Met Val Val
    180          185          190

35  Ile Pro Glu Thr Ala Gly Ala Asn Asn Thr Leu His Asn Asp Leu Cys
    195          200          205

    Thr Ala Phe Glu Glu Gly Pro Tyr Ser Thr Ile Gly Asp Asp Ala Gln
    210          215          220

40  Asp Thr Tyr Leu Ser Thr Phe Ala Gly Pro Ile Thr Ala Arg Val Asn
    225          230          235          240

    Ala Asn Leu Pro Gly Ala Asn Leu Thr Asp Ala Asp Thr Val Ala Leu
    245          250          255

45  Met Asp Leu Cys Pro Phe Glu Thr Val Ala Ser Ser Ser Ser Asp Pro
    260          265          270

    Ala Thr Ala Asp Ala Gly Gly Gly Asn Gly Arg Pro Leu Ser Pro Phe
    275          280          285

50  Cys Arg Leu Phe Ser Glu Ser Glu Trp Arg Ala Tyr Asp Tyr Leu Gln
    290          295          300

```

55

EP 0 684 313 A2

Ser Val Gly Lys Trp Tyr Gly Tyr Gly Pro Gly Asn Pro Leu Gly Pro
305 310 315 320

5 Thr Gln Gly Val Gly Phe Val Asn Glu Leu Leu Ala Arg Leu Ala Gly
325 330 335

Val Pro Val Arg Asp Gly Thr Ser Thr Asn Arg Thr Leu Asp Gly Asp
340 345 350

10 Pro Arg Thr Phe Pro Leu Gly Arg Pro Leu Tyr Ala Asp Phe Ser His
355 360 365

Asp Asn Asp Met Met Gly Val Leu Gly Ala Leu Gly Ala Tyr Asp Gly
370 375 380

15 Val Pro Pro Leu Asp Lys Thr Ala Arg Arg Asp Pro Glu Glu Leu Gly
385 390 395 400

Gly Tyr Ala Ala Ser Trp Ala Val Pro Phe Ala Ala Arg Ile Tyr Val
405 410 415

20 Glu Lys Met Arg Cys Ser Gly Gly Gly Gly Gly Gly Gly Gly Glu
420 425 430

Gly Arg Gln Glu Lys Asp Glu Glu Met Val Arg Val Leu Val Asn Asp
435 440 445

25 Arg Val Met Thr Leu Lys Gly Cys Gly Ala Asp Glu Arg Gly Met Cys
450 455 460

Thr Leu Glu Arg Phe Ile Glu Ser Met Ala Phe Ala Arg Gly Asn Gly
465 470 475 480

30 Lys Trp Asp Leu Cys Phe Ala
485

(2) INFORMATION FOR SEQ ID NO: 5:

- 35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 100 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
- 40 (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 2..100
- 45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

50 G ACC TTG GCT CGC AAC CAC ACA GAC ACG CTG TCT CCG TTC TGC GCT
 Thr Leu Ala Arg Asn His Thr Asp Thr Leu Ser Pro Phe Cys Ala
 1 5 10 15

46

55

EP 0 684 313 A2

CTT TCC ACG CAA GAG GAG TGG CAA GCA TAT GAC TAC TAC CAA AGT CTG 94
 Leu Ser Thr Gln Glu Trp Gln Ala Tyr Asp Tyr Tyr Gln Ser Leu
 20 25 30

5 GGG AAT 100
 Gly Asn

10 (2) INFORMATION FOR SEQ ID NO: 6:

 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 33 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

20 Thr Leu Ala Arg Asn His Thr Asp Thr Leu Ser Pro Phe Cys Ala Leu
 1 5 10 15
 Ser Thr Gln Glu Glu Trp Gln Ala Tyr Asp Tyr Tyr Gln Ser Leu Gly
 20 25 30
 Asn

25 (2) INFORMATION FOR SEQ ID NO: 7:

 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 106 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

35 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 2..106

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

T ACG GTA GCG CGC ACC AGC GAC GCA AGT CAG CTG TCA CCG TTC TGT 46
 Thr Val Ala Arg Thr Ser Asp Ala Ser Gln Leu Ser Pro Phe Cys
 1 5 10 15

45 CAA CTC TTC ACT CAC AAT GAG TGG AAG AAG TAC AAC TAC CTT CAG TCC 94
 Gln Leu Phe Thr His Asn Glu Trp Lys Lys Tyr Asn Tyr Leu Gln Ser
 20 25 30

50 TTG GGC AAG TAC 106
 Leu Gly Lys Tyr
 35

55

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Thr Val Ala Arg Thr Ser Asp Ala Ser Gln Leu Ser Pro Phe Cys Gln
 1 5 10 15
 Leu Phe Thr His Asn Glu Trp Lys Lys Tyr Asn Tyr Leu Gln Ser Leu
 20 25 30
 Gly Lys Tyr
 35

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 109 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 2..109

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

C ACC ATG GCG CGC ACC GCC ACT CGG AAC CGT AGT CTG TCT CCA TTT 46
 Thr Met Ala Arg Thr Ala Thr Arg Asn Arg Ser Leu Ser Pro Phe
 1 5 10 15
 TGT GCC ATC TTC ACT GAA AAG GAG TGG CTG CAG TAC GAC TAC CTT CAA 94
 Cys Ala Ile Phe Thr Glu Lys Glu Trp Leu Gln Tyr Asp Tyr Leu Gln
 20 25 30
 TCT CTA TCA AAG TAC 109
 Ser Leu Ser Lys Tyr
 35

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

EP 0 684 313 A2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Thr Met Ala Arg Thr Ala Thr Arg Asn Arg Ser Leu Ser Pro Phe Cys
 1 5 10 15
 Ala Ile Phe Thr Glu Lys Glu Trp Leu Gln Tyr Asp Tyr Leu Gln Ser
 20 25 30
 Leu Ser Lys Tyr
 35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1912 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1396

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1398

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATG GGC GTC TCT GCT GTT CTA CTT CCT TTG TAT CTC CTA GCT GGA GTC 48
 Met Gly Val Ser Ala Val Leu Leu Pro Leu Tyr Leu Leu Ala Gly Val 15
 1 5 10 15
 ACC TCC GGA CTG GCA GTC CCC GCC TCG AGA AAT CAA TCC ACT TGC GAT 96
 Thr Ser Gly Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Thr Cys Asp 20 25 30
 35
 ACG GTC GAT CAA GGG TAT CAA TGC TTC TCC GAG ACT TCG CAT CTT TGG 144
 Thr Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp 35 40 45
 40
 GGT CAA TAC GCG CCG TTC TTC TCT CTG GCA AAC GAA TCG GTC ATC TCC 192
 Gly Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser 50 55 60
 45
 CCT GAT GTG CCC GCC GGT TGC AGA GTC ACT TTC GCT CAG GTC CTC TCC 240
 Pro Asp Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser 65 70 75 80
 50
 CGT CAT GGA GCG CGG TAT CCG ACC GAG TCC AAG GGC AAG AAA TAC TCC 288
 Arg His Gly Ala Arg Tyr Pro Thr Glu Ser Lys Gly Lys Lys Tyr Ser 85 90 95
 55
 GCT CTC ATT GAG GAG ATC CAG CAG AAC GTG ACC ACC TTT GAT GGA AAA 336
 Ala Leu Ile Glu Glu Ile Gln Gln Asn Val Thr Thr Phe Asp Gly Lys 100 105 110

EP 0 684 313 A2

	TAT GCC TTC CTG AAG ACA TAC AAC TAC AGC TTG GGT GCA GAT GAC CTG	384
	Tyr Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu	
	115 120 125	
5	ACT CCC TTC GGA GAG CAG GAG CTA GTC AAC TCC GGC ATC AAG TTC TAC	432
	Thr Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr	
	130 135 140	
10	CAG CGC TAC AAC GCC CTC ACC CGA CAC ATC AAC CCC TTC GTC CGC GCC	480
	Gln Arg Tyr Asn Ala Leu Thr Arg His Ile Asn Pro Phe Val Arg Ala	
	145 150 155 160	
	ACC GAT GCA TCC CGC GTC CAC GAA TCC GCC GAG AAG TTC GTC GAG GGC	528
	Thr Asp Ala Ser Arg Val His Glu Ser Ala Glu Lys Phe Val Glu Gly	
	165 170 175	
15	TTC CAA ACC GCT CGA CAG GAC GAT CAT CAC GCC AAT CCC CAC CAG CCT	576
	Phe Gln Thr Ala Arg Gln Asp Asp His His Ala Asn Pro His Gln Pro	
	180 185 190	
20	TCG CCT CGC GTG GAC GTG GCC ATC CCC GAA GGC AGC GCC TAC AAC AAC	624
	Ser Pro Arg Val Asp Val Ala Ile Pro Glu Gly Ser Ala Tyr Asn Asn	
	195 200 205	
	ACG CTG GAG CAC AGC CTC TGC ACC GCC TTC GAA TCC AGC ACC GTC GGC	672
	Thr Leu Glu His Ser Leu Cys Thr Ala Phe Glu Ser Ser Thr Val Gly	
	210 215 220	
25	GAC GAC GCG GTC GCC AAC TTC ACC GCC GTG TTC GCG CCG GCG ATC GCC	720
	Asp Asp Ala Val Ala Asn Phe Thr Ala Val Phe Ala Pro Ala Ile Ala	
	225 230 235 240	
30	CAG CGC CTG GAG GCC GAT CTT CCC GGC GTG CAG CTG TCC ACC GAC GAC	768
	Gln Arg Leu Glu Ala Asp Leu Pro Gly Val Gln Leu Ser Thr Asp Asp	
	245 250 255	
	GTG GTC AAC CTG ATG GCC ATG TGT CCG TTC GAG ACG GTC AGC CTG ACC	816
	Val Val Asn Leu Met Ala Met Cys Pro Phe Glu Thr Val Ser Leu Thr	
	260 265 270	
35	GAC GAC GCG CAC ACG CTG TCG CCG TTC TGC GAC CTC TTC ACG GCC ACT	864
	Asp Asp Ala His Thr Leu Ser Pro Phe Cys Asp Leu Phe Thr Ala Thr	
	275 280 285	
40	GAG TGG ACG CAG TAC AAC TAC CTG CTC TCG CTG GAC AAG TAC TAC GGC	912
	Glu Trp Thr Gln Tyr Asn Tyr Leu Leu Ser Leu Asp Lys Tyr Tyr Gly	
	290 295 300	
	TAC GGC GGG GGC AAT CCG CTG GGT CCG GTG CAG GGG GTC GGC TGG GCG	960
	Tyr Gly Gly Gly Asn Pro Leu Gly Pro Val Gln Gly Val Gly Trp Ala	
	305 310 315 320	
45	AAC GAG CTG ATG GCG CGG CTA ACG CGC GCC CCC GTG CAC GAC CAC ACC	1008
	Asn Glu Leu Met Ala Arg Leu Thr Arg Ala Pro Val His Asp His Thr	
	325 330 335	
50	TGC GTC AAC AAC ACC CTC GAC GCG AGT CCG GCC ACC TTC CCG CTG AAC	1056
	Cys Val Asn Asn Thr Leu Asp Ala Ser Pro Ala Thr Phe Pro Leu Asn	
	340 345 350	

55

EP 0 684 313 A2

	GCC ACC CTC TAC GCC GAC TTC TCC CAC GAC AGC AAC CTG GTG TCG ATC	1104
	Ala Thr Leu Tyr Ala Asp Phe Ser His Asp Ser Asn Leu Val Ser Ile	
	355 360 365	
5	TTC TGG GCG CTG GGC CTG TAC AAC GGC ACC GCG CCG CTG TCG CAG ACC	1152
	Phe Trp Ala Leu Gly Leu Tyr Asn Gly Thr Ala Pro Leu Ser Gln Thr	
	370 375 380	
	TCC GTC GAG AGC GTC TCC CAG ACG GAC GGG TAC GCC GCC GCC TGG ACG	1200
10	Ser Val Glu Ser Val Ser Gln Thr Asp Gly Tyr Ala Ala Ala Trp Thr	
	385 390 395 400	
	GTG CCG TTC GCC GCT CGC GCG TAC GTC GAG ATG ATG CAG TGT CGC GCC	1248
	Val Pro Phe Ala Ala Arg Ala Tyr Val Glu Met Met Gln Cys Arg Ala	
	405 410 415	
15	GAG AAG GAG CCG CTG GTG CGC GTG CTG GTC AAC GAC CGG GTC ATG CCG	1296
	Glu Lys Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Met Pro	
	420 425 430	
	CTG CAT GGC TGC CCT ACG GAC AAG CTG GGG CGG TGC AAG CGG GAC GCT	1344
20	Leu His Gly Cys Pro Thr Asp Lys Leu Gly Arg Cys Lys Arg Asp Ala	
	435 440 445	
	TTC GTC GCG GGG CTG AGC TTT GCG CAG GCG GGC GGG AAC TGG GCG GAT	1392
	Phe Val Ala Gly Leu Ser Phe Ala Gln Ala Gly Gly Asn Trp Ala Asp	
	450 455 460	
25	TGT TTC TGATGTTGAG AAGAAAGGTA GATAGATAGG TAGTACATAT GGATTGCTCG	1448
	Cys Phe	
	465	
	GCTCTGGGTC GTTGCCCAACA ATGCATATTA CGCCCGTCAA CTGCCTTGCG CCATCCACCT	1508
30	CTCACCCCTGG ACGCAACCGA GCGGTCTACC CTGCACACGG CTTCCACCGC GACGCGCAGC	1568
	GATAAGGCGC TTTTGTACG GGGTTGGGGC TGGGGGCAGC CGGAGCCGGA GAGAGAGACC	1628
	AGCGTGAAAA ACGACAGAAC ATAGATATCA ATTCGACGCC AATTCATGCA GAGTAGTATA	1688
35	CAGACGAACT GAAACAAACA CATCACTTCC CTCGCTCCTC TCCTGTAGAA GACGCTCCCA	1748
	CCAGCCGCTT CTGGCCCTTA TTCCCGTACG CTAGGTAGAC CAGTCAGCCA GACGCATGCC	1808
	TCACAAGAAC GGGGGCGGGG GACACACTCC GCTCGTACAG CACCCACGAC GTGTACAGGA	1868
40	AAACCGGCAG CGCCACAATC GTCGAGAGCC ATCTGCAGGA ATTC	1912

(2) INFORMATION FOR SEQ ID NO: 12:

- 45 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 466 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

55

EP 0 684 313 A2

Met Gly Val Ser Ala Val Leu Leu Pro Leu Tyr Leu Leu Ala Gly Val
1 5 10 15

5 Thr Ser Gly Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Thr Cys Asp
20 25 30

Thr Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp
35 40 45

10 Gly Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser
50 55 60

Pro Asp Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser
65 70 75 80

15 Arg His Gly Ala Arg Tyr Pro Thr Glu Ser Lys Gly Lys Lys Tyr Ser
85 90 95

Ala Leu Ile Glu Glu Ile Gln Gln Asn Val Thr Thr Phe Asp Gly Lys
100 105 110

20 Tyr Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu
115 120 125

Thr Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr
130 135 140

25 Gln Arg Tyr Asn Ala Leu Thr Arg His Ile Asn Pro Phe Val Arg Ala
145 150 155 160

Thr Asp Ala Ser Arg Val His Glu Ser Ala Glu Lys Phe Val Glu Gly
165 170 175

30 Phe Gln Thr Ala Arg Gln Asp Asp His His Ala Asn Pro His Gln Pro
180 185 190

Ser Pro Arg Val Asp Val Ala Ile Pro Glu Gly Ser Ala Tyr Asn Asn
195 200 205

35 Thr Leu Glu His Ser Leu Cys Thr Ala Phe Glu Ser Ser Thr Val Gly
210 215 220

Asp Asp Ala Val Ala Asn Phe Thr Ala Val Phe Ala Pro Ala Ile Ala
225 230 235 240

40 Gln Arg Leu Glu Ala Asp Leu Pro Gly Val Gln Leu Ser Thr Asp Asp
245 250 255

Val Val Asn Leu Met Ala Met Cys Pro Phe Glu Thr Val Ser Leu Thr
260 265 270

45 Asp Asp Ala His Thr Leu Ser Pro Phe Cys Asp Leu Phe Thr Ala Thr
275 280 285

Glu Trp Thr Gln Tyr Asn Tyr Leu Leu Ser Leu Asp Lys Tyr Tyr Gly
290 295 300

50 Tyr Gly Gly Gly Asn Pro Leu Gly Pro Val Gln Gly Val Gly Trp Ala
305 310 315 320

55

EP 0 684 313 A2

Asn Glu Leu Met Ala Arg Leu Thr Arg Ala Pro Val His Asp His Thr
 325 330 335

5 Cys Val Asn Asn Thr Leu Asp Ala Ser Pro Ala Thr Phe Pro Leu Asn
 340 345 350

Ala Thr Leu Tyr Ala Asp Phe Ser His Asp Ser Asn Leu Val Ser Ile
 355 360 365

10 Phe Trp Ala Leu Gly Leu Tyr Asn Gly Thr Ala Pro Leu Ser Gln Thr
 370 375 380

Ser Val Glu Ser Val Ser Gln Thr Asp Gly Tyr Ala Ala Ala Trp Thr
 385 390 395 400

15 Val Pro Phe Ala Ala Arg Ala Tyr Val Glu Met Met Gln Cys Arg Ala
 405 410 415

Glu Lys Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Met Pro
 420 425 430

20 Leu His Gly Cys Pro Thr Asp Lys Leu Gly Arg Cys Lys Arg Asp Ala
 435 440 445

Phe Val Ala Gly Leu Ser Phe Ala Gln Ala Gly Gly Asn Trp Ala Asp
 450 455 460

25 Cys Phe
 465

(2) INFORMATION FOR SEQ ID NO: 13:

- 30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 112 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: DNA (genomic)

(x1) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

40 GACGGTCAGC CTGACCGACG ACGCGCACAC GCTGTGCGCG TTCTGCGACC TCTTCACCGC 60
 CGCCGAGTGG ACGCAGTACA ACTACCTGCT CTCGCTGGAC AAGTACTACG TC 112

(2) INFORMATION FOR SEQ ID NO: 14:

- 45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 90 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
- 50 (ii) MOLECULE TYPE: DNA (genomic)

55

EP 0 684 313 A2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

5 CAGTAACCTG GTGTCGATCT TCTGGNCGCTG GGTCTGTACA ACGGCACCAA GCCCCTGTCG 61
CAGACCACCG TGGAGGATAT CACCCGGACG 90

(2) INFORMATION FOR SEQ ID NO: 15:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

20 ATGGAYATGT GYTCNTTYGA 20

(2) INFORMATION FOR SEQ ID NO: 16:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTRCCRGCRC CRTGNCCRTA 20

(2) INFORMATION FOR SEQ ID NO: 17:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

45 TAYGCNGAYT TYTCNCAYGA 20

(2) INFORMATION FOR SEQ ID NO: 18:

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid

55

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CGRTCRTTNA CNAGNACNC

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ATGGAYATGT GYTCNTTYGA

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TTRCCRGCRG CRTGNCCRTA

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

AGTCCGGAGG TGA CTCCAGC TAGGAGATAC

55 Claims

1. A DNA sequence coding for a polypeptide having phytase activity and which DNA sequence is derived from a fungus selected from the group consisting of *Acrophialophora levis*, *Aspergillus terreus*,

Aspergillus fumigatus, Aspergillus nidulans, Aspergillus sojae, Calcarisporiella thermophila, Chaetomium rectopilum, Corynascus thermophilus, Humicola sp., Mycelia sterilia, Myrococcum thermophilum, Myceliophthora thermophila, Rhizomucor miehei, Sporotrichum cellulophilum, Sporotrichum thermophile, Scytalidium indonesicum and Talaromyces thermophilus or a DNA sequence coding for a fragment of such a polypeptide which fragment still has phytase activity.

2. A DNA sequence according to claim 1 wherein the fungus is selected from the group consisting of Acrophialophora levis, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus terreus, Calcarisporiella thermophila, Chaetomium rectopilum, Corynascus thermophilus, Sporotrichum cellulophilum, Sporotrichum thermophile, Mycelia sterilia, Myceliophthora thermophila and Talaromyces thermophilus.
3. A DNA sequence according to claim 2 wherein the fungus is selected from the group consisting of Aspergillus terreus, Myceliophthora thermophila, Aspergillus fumigatus, Aspergillus nidulans and Talaromyces thermophilus.
4. A DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence is selected from the following:
 - (a) the DNA sequence of Figure 1 [SEQ ID NO:1] or its complementary strand;
 - (b) a DNA sequence which hybridizes under standard conditions with sequences defined under (a);
 - (c) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with sequences of (a) or (b), but which codes for polypeptides having exactly the same amino acid sequences as the polypeptides encoded by these DNA sequences; and
 - (d) a DNA sequence which is a fragment of the DNA sequences specified in (a), (b) or (c).
5. A DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence is selected from the following:
 - (a) the DNA sequence of Figure 2 [SEQ ID NO:3] or its complementary strand;
 - (b) a DNA sequence which hybridizes under standard conditions with sequences defined under (a);
 - (c) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with sequences of (a) or (b), but which codes for polypeptides having exactly the same amino acid sequences as the polypeptides encoded by these DNA sequences; and
 - (d) a DNA sequence which is a fragment of the DNA sequences specified in (a), (b) or (c).
6. A DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence is selected from the following:
 - (a) a DNA sequence comprising one of the DNA sequences of Figures 4 [SEQ ID NO:5], 5 [SEQ ID NO:7], 6 [SEQ ID NO:9] or 10 [SEQ ID NO: 13 and/or SEQ ID NO:14] or its complementary strand;
 - (b) a DNA sequence which hybridizes under standard conditions with sequences defined under (a);
 - (c) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with sequences of (a) or (b) but which codes for polypeptides having exactly the same amino acid sequences as the polypeptides encoded by these DNA sequences; and
 - (d) a DNA sequence which is a fragment of the DNA sequences specified in (a), (b) or (c).
7. A DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence is selected from a DNA sequence comprising the DNA sequence of Figure 4 [SEQ ID NO:5] isolatable from Talaromyces thermophilus, of Figure 5 [SEQ ID NO:7] isolatable from Aspergillus fumigatus, of Figure 6 [SEQ ID NO:9] isolatable from Aspergillus nidulans or of Figure 10 [SEQ ID NO:13 and/or SEQ ID NO:14] isolatable from Aspergillus terreus (CBS 220.95) or which DNA sequence is a degenerate variant or equivalent thereof.
8. A DNA sequence as claimed in any one of claims 4 to 6 which codes for a polypeptide having phytase activity which DNA sequence is derived from a fungus.
9. A DNA sequence according to claim 8 wherein the fungus is selected from a group as defined in claim 1, 2 or 3.
10. A DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence hybridizes under standard conditions with a probe which is a product of a PCR reaction with DNA

isolated from a fungus as defined in any one of claims 1 to 3 and the following pair of PCR primer:

"ATGGA(C/T)ATGTG(C/T)TC(N)TT(C/T)GA" [SEQ ID NO:15] as sense primer and

"TT(A/G)CC(A/G)GC(A/G)CC(G/A)TG(N)CC(A/G)TA" [SEQ ID NO:16] as anti-sense primer.

- 5 11. A DNA sequence which codes for a polypeptide having phytase activity and which DNA sequence hybridizes under standard conditions with a probe which is a product of a PCR reaction with DNA isolated from *Aspergillus terreus* (CBS 220.95) and the following two pairs of PCR primers:
(a) "ATGGA(C/T)ATGTG(C/T)TC(N)TT(C/T)GA" [SEQ ID NO:15] as the sense primer and
"TT(A/G)CC(A/G)GC(A/G)CC(G/A)TG(N)CC(A/G)TA" [SEQ ID NO:16] as the anti-sense primer; and
10 (b) "TA(C/T)GC(N)GA(C/T)TT(C/T)TC(N)CA(C/T)GA" [SEQ ID NO: 17] as the sense primer and
"CG(G/A)TC(G/A)TT(N)AC(N)AG(N)AC(N)C" [SEQ ID NO: 18] as the anti-sense primer.
12. A DNA sequence coding for a chimeric construct having phytase activity which chimeric construct comprises a fragment of a DNA sequence as claimed in any one of claims 1 to 11.
- 15 13. A DNA sequence coding for a chimeric construct as defined in claim 12 which chimeric construct consists at its N-terminal end of a fragment of the *Aspergillus niger* phytase fused at its C-terminal end to a fragment of the *Aspergillus terreus* phytase.
- 20 14. A DNA sequence as claimed in claim 13 with the specific nucleotide sequence as shown in Figure 7 [SEQ ID NO:11] and a degenerate variant or aequivalent thereof.
15. A DNA sequence as claimed in any one of claims 1 to 14 wherein the encoded polypeptide is a phytase.
- 25 16. A polypeptide encoded by a DNA sequence as claimed in any one of claims 1 to 15.
17. A vector comprising a DNA sequence as claimed in any one of claims 1 to 15.
- 30 18. A vector as claimed in claim 17 suitable for the expression of said DNA sequence in bacteria or a fungal or a yeast host.
19. Bacteria or a fungal or yeast host transformed by a DNA sequence as claimed in any one of claims 1 to 15 or a vector as claimed in claim 17 or 18.
- 35 20. A composit food or feed comprising one or more polypeptides as defined in claim 16.
21. A process for the preparation of a polypeptide as claimed in claim 16 characterized in that transformed bacteria or host cell as claimed in claim 19 is cultured under suitable culture conditions and the polypeptide is recovered therefrom.
- 40 22. A polypeptide when produced by a process as claimed in claim 21.
23. A process for the preparation of a composit feed or food wherein the components of the composition are mixed with one or more polypeptides as defined in claim 16.
- 45 24. A process for the reduction of levels of phytate in animal manure characterized in that an animal is fed a composit feed as defined in claim 20 in an amount effective in converting phytate contained in the feedstuff to inositol and inorganic phosphate.
- 50 25. Use of a polypeptide according to claim 16 for the conversion of phytate to inositol phosphates, inositol and inorganic phosphate.

Fig. 1/1

tctagaacaataacaggtactccctaggtacccgaaggaccttgtggaaaatgtatggag 60
 gtggacacggcaccacaccacccgcgatggcgacgtgggtgccctaacccttgtctcc 120
 ctgaggtggaatccatgtcgactctttaccctcaccatcgccctggatgaaacctccccg 180
 ctaagctcacgacgatcgctatttccgaccgatttgaccgtcatgggtggagggtgattc 240
 ggtcgatgctcctgccttcatttcggagttcgagacatgaaaggcttatatgaggacgt 300
 cccaggtcggggacgaaatccgcctgggtgtgctccttcgtcggaacatctgctgtc 360
 cgtgatggctaccatgggctttcttgccattgtgctctccgtcgcccttgcctttagaag 420
 M G F L A I V L S V A L L F R S 16
 gtatgcaccctctacgtccaattctctgggcactgacaacggcgacacatcggggcac 480
 T S G T 20
 cccgttgggccccggggcacaacatagcgactgcaactcagtcgatcacggctatcaatg 540
 P L G P R G K H S D C N S V D H G Y Q C 40
 ctttcctgaactctctcataaatggggactctacgcgccctacttctccctccaggacga 600
 F P E L S H K W G L Y A P Y F S L Q D E 60
 gtctccgtttcctctggacgtcccagaggactgtcacatcaccttcgtgcaggtgctggc 660
 S P F P L D V P E D C H I T F V Q V L A 80
 ccggcacggcgcgcgaggcccaaccatagcaagaccaaggcgtagcgggcgaccattgc 720
 R H G A R S P T H S K T K A Y A A T I A 100
 ggccatccagaagagtgcactgcgtttccgggcaaatacgcgttcctgcagtcataaa 780
 A I Q K S A T A F P G K Y A F L Q S Y N 120
 ctactccttgactctgaggagctgactcccttcgggcggaaccagctgcgagatctggg 840
 Y S L D S E E L T P F G R N Q L R D L G 140
 cgcccagttctacgagcgctacaacgccctcaccgcacacatcaacccttcgtccgcgc 900
 A Q F Y E R Y N A L T R H I N P F V R A 160
 caccgatgcaccccgctccacgaatccgcccagagaagttcgtcgagggcttccaaaccgc 960
 T D A S R V H E S A E K F V E G F Q T A 180
 tcgacaggacgatcatcacgccaatccccaccagccttcgcctcgcgtggacgtggccat 1020
 R Q D D H H A N P H Q P S P R V D V A I 200
 cccgaaggcagcgccctacaacaacacgctggagcacagcctctgcaccgccttcgaatc 1080
 P E G S A Y N N T L E H S L C T A F E S 220
 cagcaccgctcgcgacgacgcgggtcgccaacttcaccgcgcgtgttcgcgcggcgatcgc 1140
 S T V G D D A V A N F T A V F A P A I A 240
 ccagcgccctggaggcgatcttcccgcggtgcagctgtccaccgacgacgtggtcaacct 1200
 Q R L E A D L P G V Q L S T D D V V N L 260
 gatggccatgtgtccgttcgagacgggtcagcctgaccgacgacgcgcacacgctgtcgcc 1260
 M A M C P F E T V S L T D D A H T L S P 280
 gttctgcgacctcttcacggccactgagtgacgcagtagaactacctgctctcgctgga 1320
 F C D L F T A T E W T Q Y N Y L L S L D 300
 caagtactacggctacggcgggggcaatccgctgggtccggtgcagggggtcggtgggc 1380
 K Y Y G Y G G G N P L G P V Q G V G W A 320
 gaacgagctgatggcgcggttaacgcgcgccccgtgcacgaccacacctgcgtcaacaa 1440
 N E L M A R L T R A P V H D H T C V N N 340
 caccctcgacgcgagtcggccaccttcccgctgaacgccacctctacgccgacttctc 1500
 T L D A S P A T F P L N A T L Y A D F S 360

Fig. 1/2

ccacgacagcaacctggtgtcgatcttctgggcgctgggcctgtacaacggcaccgcgcc 1560
 H D S N L V S I F W A L G L Y N G T A P 380

 gctgtcgcagacctccgtcgagagcgtctcccagacggacgggtacgccgcgcctggac 1620
 L S Q T S V E S V S Q T D G Y A A A W T 400

 ggtgccgttcgccgctcgcgcgtacgtcgagatgatgcagtgtcgcgccgagaaggagcc 1680
 V P F A A R A Y V E M M Q C R A E K E P 420

 gctggtgcgcgtgctggtcaacgacgggtcatgccgctgcatggctgccctacggacaa 1740
 L V R V L V N D R V M P L H G C P T D K 440

 gctggggcggtgcaagcgggacgctttcgtcgcggggctgagctttgcgcaggcggggcg 1800
 L G R C K R D A F V A G L S F A Q A G G 460

 gaactgggcggattgtttctgatgttgagaagaaaggtagatagataggtagtacatatg 1860
 N W A D C F 466

 gattgctcggctctgggtcgttggccacaatgcatattacgcccggtcaactgccttgccg 1920
 catccacctctcaccctggacgcaaccgagcgggtctaccctgcacacggcttcaccgcg 1980
 acgcgcacggataaggcgcttttggttacggggttggggctgggggcagccggagccggag 2040
 agagagaccagcgtgaaaaacgacagaacatagatatcaattcgacgccaattcatgcag 2100
 agtagtatacagacgaactgaaacaaacacatcacttccctcgctcctctcctgtagaag 2160
 acgctcccaccagccgcttctggcccttattcccgtacgctaggtagaccagtcagccag 2220
 acgcatgcctcacaagaacggggggggggacacactccgctcgtacagcaccacgacg 2280
 tgtacaggaaaaccggcagcgcacaaatcgtcgagagccatctgcag 2327

Fig. 2/1

gtcgacgagggcacaccacgcccgtcctcggcggggtccgagagggccggggtcgggttcga 60
 caaggagacggggtcccttcggggcgcggtgcgggtgtgggtgttgcgtgtggacgggtga 120
 ggagggggacggggtggcggtgatgacgggtacgaatgcgaacggacacagggcggtgag 180
 cgtgggtgttgcgttctaattcttcttctgtgtgggtgtgtacgtgtgggtgtgtatgtgt 240
 ttgggggggggaatgttcttggtaattatcttctacccttcttctcttcttcttattct 300
 gttcagcaggtatacccggtgtaagtgtacaggattatgggacgggtgggtggatggact 360
 acttctagaaggacggataaaggaaaaaggggaaacacgaatatggcgccctgggtggcgc 420
 gtcgagctggatgcttgacgcccgtctggcaaacatttcttcttcttagcaccacaaccta 480
 gtacttgatagagtgttccggggccaggcggtttgcgtgtgtttttaccaatcaccaac 540
 tagtgctactactattattgcggtgtgtatgcagccgtgtacaaaaaatgccgcgcat 600
 ctccattgatacttgtagttttgatagatcaatatgtgggaggttgcgtgggtgtctct 660
 gaaacccctctctcttgcgtgtacgtatgtgcacagatgtcaccgacaaagacga 720
 ttgcatgcgcatcgtttttgtgtgtttcaggcctcgtcgtgtctagggtataaacac 780
 attgaagactacatatgcgcaagcgttgacattaacgggggtcctgcagccgcccaggt 840
 gcatgtcgtgattaataccacgcgctgcgtaaattagctagccgcccctgtttcact 900
 cgggttagagacggacaggtgagacgggtctcgggttaagcaagcaaatgggaatgcaagg 960
 tgaagggtgaatctgcatagcgtgaaatgagaggggtcctgtgggcagccaggaagggtga 1020
 gacgaaatgaggaagaggcaccagaagctgttgttctgaagtcccgtggtcatagctc 1080
 caggattaaagtacggatgtcccatgccaagctgctggcttcgaaagcagtagcggagtag 1140
 tgtccattgttcacgagggatccccaatgtgttagacatgcctgaatcaattttgtccta 1200
 tttttggatttcaactgtttctctcgactgtgctcggtagcagctatgccgcaaggtaga 1260
 ctacatgttgtacaataatcatacatcgacctccgtaggagtgctgaaatacccgacct 1320
 gctctctctagcaggtgcctaattggtttcgtgttaactcgatcgaaacggatcagcaagt 1380
 ccatttgcgtgtgggtgagatgtacgatttacaacacgtggagaggtgagccacagcga 1440
 taggcttctggaaggattctggcgtctcggaagaggggccactcgccccactaacggcg 1500
 ccgatcttgacatggggtcgcaggggtttaagtgcacactacggagtagcggattacac 1560
 agtagtgtatgggtggggcgagttgggtggccttgtgtggggtcaccgggtgcctgt 1620
 tctcggggaggtcttggcgggccgattggaccacctaaccacgggtagcttggcccggc 1680
 caactcacaccgcccctcatgttccggagccagtcagggagggcagggcactactcagtcagg 1740
 tacacacgtcgggctcctcgatgctgggtgacatcgaggcgatctgcattccaactacg 1800
 gttggcataggaggtatcctattctagagctgttctacgccggaacgtaaccgggataa 1860
 cccgggatatcgcttccctgagcagcgcgctgctgaggatcatacaacccaacaaccga 1920
 cgacggtgcaagaaggttgggggaaggaagaaatcaaggaaaaaaatagggggggtgg 1980
 ggaccaagagagaaagaaaggagaaaagggtggggggaggggaagagaaaaaaaacgga 2040
 ggaatatggcgctcgtcttccgactggttccggaagggggcatctgggtacacatatgcac 2100
 ctcttccgcacggcagggatataaaccgggagtgacgtcccacggatcatgctgagtcg 2160
 cccgtctccagacttcacgggtcgcagaggactagacgcggtgaagatgactggcctcg 2220
 M T G L G 5

gagtgatgggtgatgggtcggcttccctggcgatcgctctctgtgaagcagcgattccag 2280
 V M V V M V G F L A I A S L 19

gggtccggtgtgcgttaaaagaaaaagctaaccgccaccagacaatccgagtcggggccat 2340
 Q S E S R P C 26

gcgacacccccagacttgggcttccagtgtggtacggccatttcccacttctggggccagt 2400
 D T P D L G F Q C G T A I S H F W G Q Y 46

actcgccctacttctccgtgccctcggagctggatgcttccgatccccgacgactgagagg 2460
 S P Y F S V P S E L D A S I P D D C E V 66

tgacgtttgcccagtcctctcccgccacggcgagggcgccgacgctcaaacggggccg 2520
 T F A Q V L S R H G A R A P T L K R A A 86

cgagctacgtcgatctcatcgacaggatccaccatggcgccatctcctacgggcccgggt 2580
 S Y V D L I D R I H H G A I S Y G P G Y 106

acgagttcctcaggacgtatgactacaccctggcgccgacgagctcaccgggacggggc 2640
 E F L R T Y D Y T L G A D E L T R T G Q 126

agcagcagatgggtcaactcgggcatcaagttttaccgcccgtaccgcgctctcggccgca 2700
 Q Q M V N S G I K F Y R R Y R A L A R K 146

Fig. 2/2

```

agtcgatcccccttcgtccgcaccgcccggccaggaccgcgtcgtccactcggccgagaact 2760
S I P F V R T A G Q D R V V H S A E N F 166

tcacccagggcttcactctgccctgctcggcgaccgcggtccaccgtccggcccaccc 2820
T Q G F H S A L L A D R G S T V R P T L 186

tcccctatgacatggctcgtcatcccggaaaccgcccggcgccaacaacacgctccacaacg 2880
P Y D M V V I P E T A G A N N T L H N D 206

acctctgcaccgccttcgaggaaggcccgtactcgaccatcggcgacgacgcccgaagaca 2940
L C T A F E E G P Y S T I G D D A Q D T 226

cctacctctccaccttcgccggacccatcacgcgccgggtcaacgccaacctgccggggcg 3000
Y L S T F A G P I T A R V N A N L P G A 246

ccaacctgaccgacgcccacacggctcgcgctgatggacctctgccccttcgagacggctcg 3060
N L T D A D T V A L M D L C P F E T V A 266

cctcctcctcctccgacccggcaacggcgacgcgggggcggaacggcgcgccgctgt 3120
S S S S D P A T A D A G G G N G R P L S 286

cgcccttctgccgctgttcagcgagtcgagtgccgcgctacgactacctgcagtcgg 3180
P F C R L F S E S E W R A Y D Y L Q S V 306

tgggcaagtggtagcgggtacgggcccgggcaacccgctggggccgacgcagggggtcggt 3240
G K W Y G Y G P G N P L G P T Q G V G F 326

tcgtcaacgagctgctggcgcggtggccgggtccccgtgcgcgacggcaccagcacca 3300
V N E L L A R L A G V P V R D G T S T N 346

accgcaccctcgacggcgacccgcgcaccttcccgtcggccggccccctctacgccgact 3360
R T L D G D P R T F P L G R P L Y A D F 366

tcagccacgacaacgacatgatggcgctcctcgggccttcggcgccctacgacggcgctcc 3420
S H D N D M M G V L G A L G A Y D G V P 386

cgccccctcgacaagaccgcccgcgcgacccggaagagctcggcgggtagcgggccagct 3480
P L D K T A R R D P E E L G G Y A A S W 406

gggcccgtcccgttcgccgccaggatctacgtcgagaagatgcggtgcagcgggcgggcg 3540
A V P F A A R I Y V E K M R C S G G G G 426

gcggcgggcgggcgggcgagggggcgaggaaggatgaggagatggtcaggggtgctgg 3600
G G G G G E G R Q E K D E E M V R V L V 446

tgaacgaccgggtgatgacgctgaaggggtcgggcgccgacgagagggggatgtgtacgc 3660
N D R V M T L K G C G A D E R G M C T L 466

tagaacggttcacgaaagcatggcggtttcgaggggggaacggcaagtgggatctctgct 3720
E R F I E S M A F A R G N G K W D L C F 486

ttgcttgatatgccacgcccagattgaacagaacttgatgaggggtagagtgtggta 3780
A 487

ttcgagatgatagttcacagttttcggaatcaaaaatcggttagactggcgaaattcaa 3840
gtctggggcctgcccgtctgcattctccgttccctgttggttaccttcttaattggtttt 3900
ttttattttttatttttcttaaattttcacacaaaccttttattgtctttttttcttctt 3960
tttcttctctgcacatcgatgggaattgtcgac 3995

```

Fig. 3A

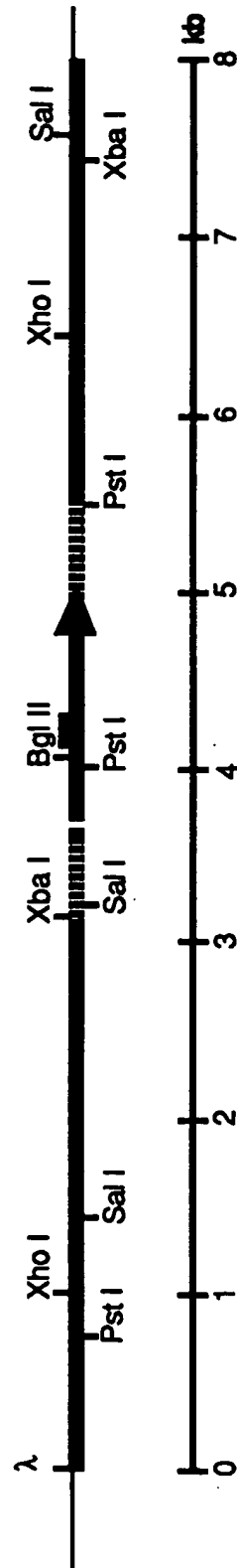


Fig. 3B

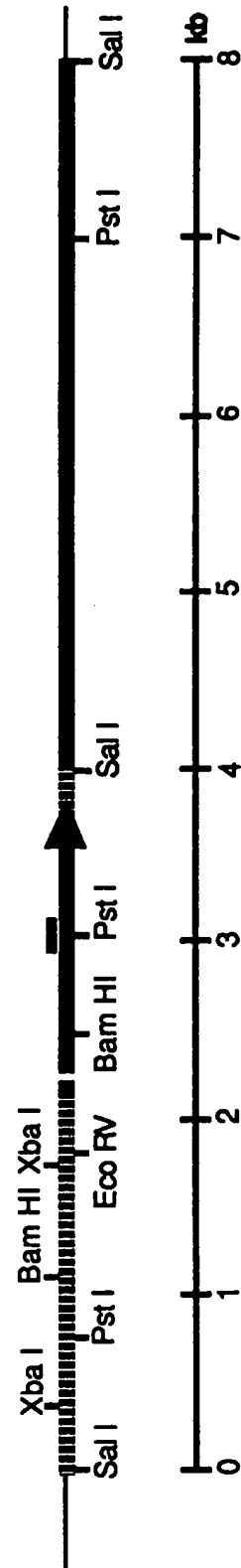


Fig. 4

```

gaccttggctcgcaaccacacagacacgctgtctccgttctgcgctctttccacgcaaga
1 -----+-----+-----+-----+-----+ 60
ctggaaccgagcgttggtgtgtctgtgcgacagaggcaagacgcgagaaaggtgcgttct

  T  L  A  R  N  H  T  D  T  L  S  P  F  C  A  L  S  T  Q  E

ggagtggcaagcatatgactactaccaaagtctggggaat
61 -----+-----+-----+-----+ 100
cctcaccgttcgtatactgatgatggtttcagacccttt

  E  W  Q  A  Y  D  Y  Y  Q  S  L  G  N

```

Fig. 5

```

tacggtagcgcgcaccagcgcgcaagtcagctgtcacccgttctgtcaactcttcactca
1 -----+-----+-----+-----+-----+-----+ 60
atgccatcgcgcgtggtcgctgcgttcagtcgcacagtggcaagacagttgagaagtgagt
  T V A R T S D A S Q L S P F C Q L F T H

caatgagtgggaagaagtacaactaccttcagtccttgggcaagtac
61 -----+-----+-----+-----+-----+ 106
gttactcaccttcttcattgatggaagtcaggaacccggttcattg
  N E W K K Y N Y L Q S L G K Y

```


Fig. 6

```

caccatggcgcgccacgcccactcggaaccgtagtctgtctccattttgtgccatcttcac
1 -----+-----+-----+-----+-----+-----+-----+ 60
gtggtaccgcgcggtggcggtgagccttggcatcagacagaggtaaaacacggtagaagtg
  T M A R T A T R N R S L S P F C A I F T

tgaaaaggagtggtgcagtagcactaccttcaatctctatcaaagtac
61 -----+-----+-----+-----+-----+-----+ 109
acttttcctcaccgacgtcatgctgatggaagttagagatagtttcatg
  E K E W L Q Y D Y L Q S L S K Y

```

Fig. 7/1

```

atgggcgtctctgctgttctacttcctttgtatctcctagctggagtcacctccggactg
1  -----+-----+-----+-----+-----+-----+ 60
taccgcgagagacgacaagatgaaggaaacatagaggatcgacctcagtgaggcctgac
M G V S A V L L P L Y L L A G V T S G L

gcagtcctccgcctcgagaaatcaatccacttgcgatacggtcgatcaagggtatcaatgc
61  -----+-----+-----+-----+-----+-----+ 120
cgtcagggggcgagctcttttagttaggtgaacgctatgccagctagttcccatagttacg
A V P A S R N Q S T C D T V D Q G Y Q C

ttctccgagacttcgcatctttggggtaatacgcgcggttcttctctctggcaaacgaa
121 -----+-----+-----+-----+-----+-----+ 180
aagaggctctgaagcgtagaaacccagttatgcgcggcaagaagagagaccgtttgctt
F S E T S H L W G Q Y A P F F S L A N E

tcggtcacatctccctgatgtgccgcggttgacagagtcactttcgctcaggtcctctcc
181 -----+-----+-----+-----+-----+-----+ 240
agccagtagaggggactacacggggcgccaacgtctcagtgaaagcgagtcaggagagag
S V I S P D V P A G C R V T F A Q V L S

cgtcagggagcgcggtatccgaccgagtcgaagggaagaataactccgctctcattgag
241 -----+-----+-----+-----+-----+-----+ 300
gcagtacctcgcgccataggtggctcaggttcccgttctttatgaggcgagagtaactc
R H G A R Y P T E S K G K K Y S A L I E

gagatccagcagaacgtgaccacctttgatggaaaatatgccttcctgaagacatacaac
301 -----+-----+-----+-----+-----+-----+ 360
ctctaggtcgtcttgactgggtggaaactaccttttatacgggaaggacttctgtatgttg
E I Q Q N V T T F D G K Y A F L K T Y N

tacagcttgggtgcagatgacctgactcccttcggagagcaggagctagtcaactccggc
361 -----+-----+-----+-----+-----+-----+ 420
atgtcgaaccacgtctactggactgaggggaagcctctcgtcctcgatcagttgaggccg
Y S L G A D D L T P F G E Q E L V N S G

atcaagttctaccagcgctacaacgcctcaccgcacacatcaacccttcgtccgcgcc
421 -----+-----+-----+-----+-----+-----+ 480
tagttcaagatggtcgcatgttgctgggagtggtgtgtagttggggaagcaggcgcg
I K F Y Q R Y N A L T R H I N P F V R A

accgatgcatcccgctccacgaatccgccgagaagtctcgtcaggggcttccaaaccgct
481 -----+-----+-----+-----+-----+-----+ 540
tggctacgtagggcgaggtgcttaggcggctcttcaagcagctcccgaagggttgccga
T D A S R V H E S A E K F V E G F Q T A

cgacaggacgatcatcacgccaatccccaccagccttcgcctcgcgtggacgtggccatc
541 -----+-----+-----+-----+-----+-----+ 600
gctgtcctgctagtagtgcggttaggggtggtcggaagcggagcgacctgcaccggtag
R Q D D H H A N P H Q P S P R V D V A I

```

Fig. 7/2

```

cccgaaggcagcgcctacaacaacacgctggagcacagcctctgcaccgccttcgaatcc
601 -----+-----+-----+-----+-----+-----+ 660
gggcttccgtcgcggatgttgtgtgtgcgacctcgtgtcggagacgtggcggaagcttagg
P E G S A Y N N T L E H S L C T A F E S

agcaccgtcggcgacgacgcgggtcgccaacttcaccgccgtgttcgcgccggcgatcgcc
661 -----+-----+-----+-----+-----+-----+ 720
tcgtggcagccgctgctgcgccagcggttgaagtggcggcacaagcgcgccgctagcgg
S T V G D D A V A N F T A V F A P A I A

cagcgcctggaggccgatcttcccggcgtgcagctgtccaccgacgacgtggtcaacctg
721 -----+-----+-----+-----+-----+-----+ 780
gtcgcggacctccggctagaagggccgcacgtcgacaggtggctgctgcaccagttggac
Q R L E A D L P G V Q L S T D D V V N L

atggccatgtgtccgttcgagacgggtcagcctgaccgacgacgcgcacacgctgtcgccg
781 -----+-----+-----+-----+-----+-----+ 840
taccggtacacaggcaagctctgccagtcggactggctgctgcgcgtgtgcgacagcggc
M A M C P F E T V S L T D D A H T L S P

ttctgcgacctcttcacggccactgagtgagcagtagtacaactacctgctctcgctggac
841 -----+-----+-----+-----+-----+-----+ 900
aagacgctggagaagtgccggtgactcacctgcgtcatgttgatggacgagagcgacctg
F C D L F T A T E W T Q Y N Y L L S L D

aagtactacggctacggcgggggcaatccgctgggtccggtgcagggggtcggtgggcg
901 -----+-----+-----+-----+-----+-----+ 960
ttcatgatgccgatgccgcccccgtaggcgacccaggccacgtccccagccgacccgc
K Y Y G Y G G G N P L G P V Q G V G W A

aacgagctgatggcgcggttaacgcgcgcccccggtgcacgaccacacctgcgtcaacaac
961 -----+-----+-----+-----+-----+-----+ 1020
ttgctcgactaccgcgccgattgcgcgcgggggcacgtgctgggtgtggacgcagttgttg
N E L M A R L T R A P V H D H T C V N N

acctcgcagcgcgagtcgggccaccttcccgtgaacgccaccctctacgccgacttctcc
1021 -----+-----+-----+-----+-----+-----+ 1080
tgggagctgcgctcaggccggtggaagggcgacttgcggtgggagatgcggctgaagagg
T L D A S P A T F P L N A T L Y A D F S

cacgacagcaacctgggtgtcgatcttctgggcgctgggcctgtacaacggcaccgcgccg
1081 -----+-----+-----+-----+-----+-----+ 1140
gtgctgtcgttgaccacagctagaagaccgcgacccggacatgttgccgtggcgcgggc
H D S N L V S I F W A L G L Y N G T A P

ctgtcgcagacctccgtcgagagcgtctcccagacggacgggtacgccgccgctggacg
1141 -----+-----+-----+-----+-----+-----+ 1200
gacagcgtctggaggcagctctcgagaggggtctgcctgcccatgcggcgccgacctgc
L S Q T S V E S V S Q T D G Y A A A W T

```

Fig. 7/3

```

gtgccgttcgccgctcgcgcgtacgtcgagatgatgcagtgtcgcgccgagaaggagccg
1201 -----+-----+-----+-----+-----+-----+ 1260
cacggcaagcggcgagcgcgcgtacgtctactacgtcacagcgcggctcttcctcggc
V P F A A R A Y V E M M Q C R A E K E P

ctggcgcggtgctggtcaacgaccgggtcatgccgctgcatggctgccctacggacaag
1261 -----+-----+-----+-----+-----+-----+ 1320
gaccacgcgcacgaccagttgctggcccagtagcggcgacgtaccgacgggatgcctgttc
L V R V L V N D R V M P L H G C P T D K

ctggggcggtgcaagcgggacgctttcgtcgggggctgagctttgcgcagggcgggcg
1321 -----+-----+-----+-----+-----+-----+ 1380
gaccccgccacgttcgcctcgcaaagcagcgccccgactcgaaacgcgtccgccccgcc
L G R C K R D A F V A G L S F A Q A G G

aactggcggtattgtttctgatgttgagaagaaaggtagatagataggtagtagcatatgg
1381 -----+-----+-----+-----+-----+-----+ 1440
ttgaccgcgctaacaagactacaactcttctttccatctatctatccatcatgtatacc
N W A D C F

attgctcggctctgggtcggtgcccacaatgcatattacgcccgtcaactgccttgcgcc
1441 -----+-----+-----+-----+-----+-----+ 1500
taacgagccgagaccagcaacgggtgttacgtataatgcgggcagttgacggaacgcgg

atccacctctcaccctggacgcaaccgagcgggtctaccctgcacacggcttcaccgcga
1501 -----+-----+-----+-----+-----+-----+ 1560
taggtggagagtgggacctgcgttggtcgccagatgggacgtgtgccgaaggtggcgct

cgcgcacgggataaggcgcttttggtacgggggtggggctgggggcagccggagccggaga
1561 -----+-----+-----+-----+-----+-----+ 1620
gcgcgtgcctattccgcgaaaacaatgcccccaaccccgacccccgtcggcctcggcctct

gagagaccagcgtgaaaaacgacagaacatagatatcaattcgacgccaattcatgcaga
1621 -----+-----+-----+-----+-----+-----+ 1680
ctctctggtcgccactttttgctgtcttgtagtatatagttaagctgcggttaagtagctct

gtagtatacagacgaaactgaaacaaacacatcacttccctcgctcctctcctgtagaaga
1681 -----+-----+-----+-----+-----+-----+ 1740
catcatatgtctgcttgactttgtttgtgtagtgaagggagcgaggagaggacatcttct

cgctcccaccagcgttctggcccttattcccgtagcgttaggttagaccagtcagccaga
1741 -----+-----+-----+-----+-----+-----+ 1800
gcgaggggtggtcggcgaagaccgggaataagggcatgcgatccatctggtcagtcggtct

cgcatgcctcacaagaacggggcgggggacacactccgctcgtagcagcaccacgacgt
1801 -----+-----+-----+-----+-----+-----+ 1860
gcgtacggagtgttcttgccccgccccctgtgtgagggagcatgtcgtgggtgctgca

gtacaggaaaaccggcagcgccacaatcgtagagccatctgcaggaattc
1861 -----+-----+-----+-----+-----+-----+ 1912
catgtccttttgccgctcgcggtgttagcagctctcggtagacgtccttaag

```

Fig. 8

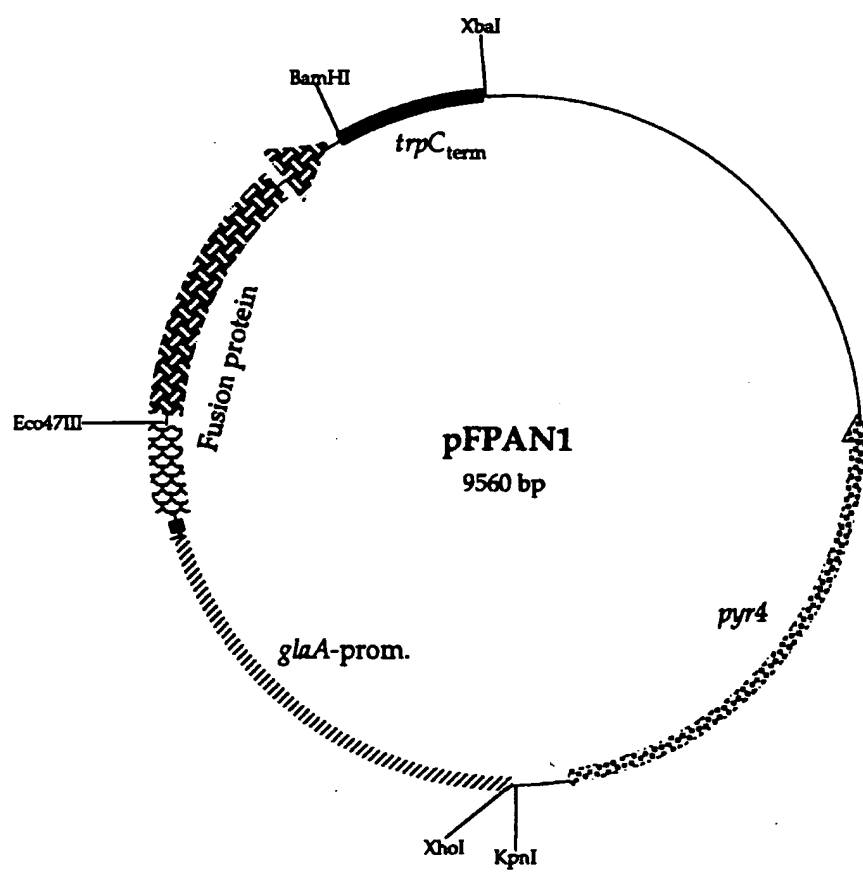
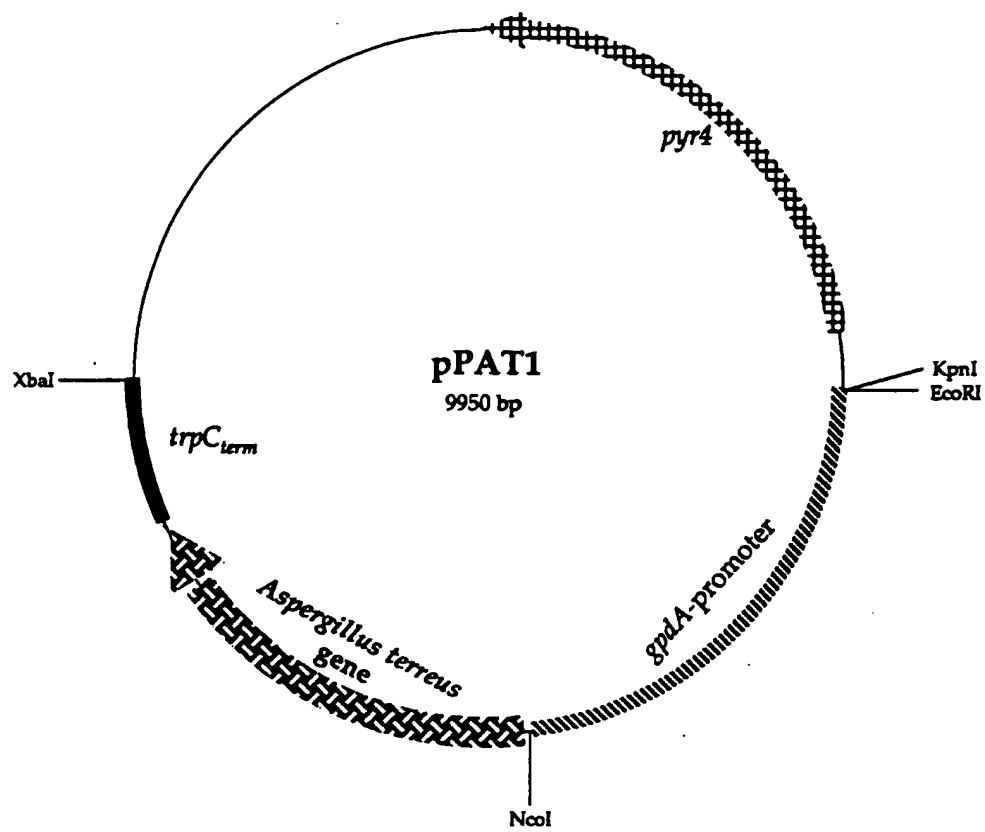


Fig. 9



```

9a1      1222  gacgggtcagcctgaccgacgacgcgcacacgctgtcgccgttctgcgacc 1271
aterr21   1  gacgggtcagcctgaccgacgacgcgcacacgctgtcgccgttctgcgacc 50

9a1      1272  tcttcacggccactgagtggacgcagtagacaactacctgctctcgctggac 1321
aterr21   51  tcttcacggccactgagtggacgcagtagacaactacctgctctcgctggac 100

9a1      1322  aagtactacggc 1333
          |||||
aterr21  101  aagtactacgtc 112

```

9a1 1507 cagcaacctgggtgtcgatcttctgggcgcctgggcctgtacaacggcaccg 1556
 ||| | | | | | | | | | |
aterr58 1 cagtaacctgggtgtcgatcttctggxcgcctgggtctgtacaacggcacca 50
9a1 1557 cgccgctgtgcgagacctccgtcgagagcgtctcccagaçg 1597
 ||| | | | | | | | | | |
aterr58 51 agccccgtgcgagaccaccgtggaggatatcaccçggacg 91



(12) **EUROPEAN PATENT APPLICATION**

(88) Date of publication A3:
19.05.1999 Bulletin 1999/20

(51) Int. Cl.⁶: **C12N 15/55**, C12N 9/16,
A23K 1/165

(43) Date of publication A2:
29.11.1995 Bulletin 1995/48

(21) Application number: 95105693.6

(22) Date of filing: 15.04.1995

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB IT LI NL

(30) Priority: 25.04.1994 EP 94810228

(71) Applicant:
F. Hoffmann-La Roche AG
4002 Basel (CH)

(72) Inventors:
• Van Loon, Adolphus
CH-4310 Rheinfelden (CH)
• Mitchell, David
CH-4147 Aesch (CH)

(74) Representative: Braun, Axel et al
F.Hoffmann-La Roche AG
Patent Department (PLP),
124 Grenzacherstrasse
4070 Basel (CH)

(54) **Polypeptides with phytase activity**

(57) The present invention is directed to a DNA sequence coding for a polypeptide having phytase activity which DNA sequence is derived from specific groups of fungi, polypeptides encoded by such DNA sequences, vectors comprising such DNA sequences, bacteria or a fungal or yeast host transformed by such DNA sequences or vectors, a process for the preparation of a polypeptide by culturing such transformed hosts and composite feeds comprising one or more such polypeptides.



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 95 10 5693

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,X	YAMADA K ET AL: "PHYTASE FROM ASPERGILLUS TERREUS. PART I. PRODUCTION, PURIFICATION AND SOME GENERAL PROPERTIES OF THE ENZYME" AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 32, no. 10, July 1968, pages 1275-1282, XP002053182	1-3,16,22	C12N15/55 C12N9/16 A23K1/165
Y	* the whole document *	4-15,17-21,23-25	
X	--- PIDDINGTON C S ET AL: "The cloning and sequencing of the genes encoding phytase (phy) and pH 2.5-optimum acid phosphatase (aph) from Aspergillus niger var awamori" GENE, vol. 133, 1993, pages 55-62, XP002089982 * the whole document *	4-8	
Y	--- MULLANEY EJ ET AL: "POSITIVE IDENTIFICATION OF A LAMBDA GT11 CLONE CONTAINING A REGION OF FUNGAL PHYTASE GENE BY IMMUNOPROBE AND SEQUENCE VERIFICATION" APPL MICROBIOL BIOTECHNOL, vol. 35, no. 5, August 1991, pages 611-614, XP002096740 * the whole document *	4-15,17-19,21	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N A23K
Y	--- PEN J ET AL: "PHYTASE-CONTAINING TRANSGENIC SEEDS AS A NOVEL FEED ADDITIVE FOR IMPROVED PHOSPHORUS UTILIZATION" BIO/TECHNOLOGY, vol. 11, no. 7, July 1993, pages 811-814, XP002026203 * the whole document *	20,23-25	
	--- -/--		
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 22 March 1999	Examiner Bilang, J
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document</p> <p>T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons &: member of the same patent family, corresponding document</p>			

EPO FORM 1503 03/82 (P04C01)



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 95 10 5693

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,A	SEGUEILHA L ET AL: "PURIFICATION AND PROPERTIES OF THE PHYTASE FROM SCHWANNIOMYCES CASTELLII" JOURNAL OF FERMENTATION AND BIOENGINEERING, vol. 74, no. 1, 1992, pages 7-11, XP002053183 * the whole document *		
L	MITCHELL DB ET AL: "THE PHYTASE SUBFAMILY OF HISTIDINE ACID PHOSPHATASES: ISOLATION OF GENES FOR TWO NOVEL PHYTASES FROM THE FUNGI ASPERGILLUS TERREUS AND MYCELIOPHTHORA THERMOPHILA" MICROBIOLOGY, vol. 143, 1997, pages 245-252, XP002097277 * the whole document *		
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 22 March 1999	Examiner Bilang, J
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03/92 (P04C01)